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Role of Heme Compounds and Haptoglobin in *Vibrio vulnificus* Pathogenicity

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An induced peritonitis model was employed in mice to determine whether heme-containing molecules enhance the lethality of infections by *Vibrio vulnificus*. The lethality of intraperitoneal (ip) inocula of the bacteria was increased by concurrent injections (ip) of hemoglobin, methemoglobin, or hematin, but not by myoglobin. Similar results were obtained in mice with phenylhydrazine-induced hemoglobinemia, in which after ip injections of *V. vulnificus*, a direct correlation between lethality and levels of plasma hemoglobin was observed. In vitro studies indicated that the growth of *V. vulnificus*, which was limited in an iron-poor medium, was enhanced by the addition of hemoglobin in a manner similar to an inorganic iron source, ferric ammonium citrate. These results suggest that *V. vulnificus* is capable of extracting iron from hemoglobin for use as a nutrilite, thereby promoting growth and increased lethality in the in vivo models. Further studies with human serum cultures demonstrated that the growth of *V. vulnificus* was not decreased when hemoglobin added to the serum was completely complexed with haptoglobin; these results are in opposition to those with cultures of *Escherichia coli*. These results are discussed relative to the capacity of *V. vulnificus* to produce fatal human infections.

The majority of iron (Fe) in mammalian fluids is tightly bound with Fe-binding proteins such as transferrin and lactoferrin. Bacteria which multiply in these fluids with low concentrations of free Fe, therefore, must possess mechanisms for assimilating protein-bound Fe or for acquiring Fe from heme-containing compounds. The acquisition of protein-bound iron by bacteria has been studied extensively. In summary, many microorganisms produce low-molecular-weight chelators, termed siderophores, which bind Fe (12, 13, 15, 17, 19). These compounds are then transported across the lipid-protein boundary by membrane-bound transport proteins which are only synthesized during times of Fe limitation (3–5). Much less information, however, is available on the utilization of Fe from heme. Recent studies have demonstrated that *Escherichia coli* exhibits enhanced pathogenicity and growth in the presence of hemoglobin (Hb) in a manner similar to that facilitated by Fe-equivalent amounts of a noneheme Fe, ferric ammonium citrate (FAC) (7, 11). In addition, a number of *Neiserria* species are apparently able to acquire Fe from hemin, but some of these species are unable to obtain Fe from Hb (14).

Among other bacteria whose pathogenesis and acquisition of Fe have been characterized is *Vibrio vulnificus*, an extremely virulent bacterium associated with septicemia leading to an unusually large proportion of cases resulting in death (1). Infections caused by this bacterium present two distinct clinical syndromes, depending on the portal of entry. Although the fatality rate after wound infections is not great, ingestion of *V. vulnificus* (primarily during the consumption of raw oysters) results in primary septicemia, with 46% of these infections proving fatal (1). A significant factor associated with these infections is that 75% of the cases had preexisting hepatic dysfunctions, hemochromatosis, or thalassemia, syndromes which may result in Fe overload (1). In vivo and in vitro studies by Wright et al. (22) directly correlate the pathogenicity of *V. vulnificus* with the availability of Fe. Although normal human serum is bactericidal for *V. vulnificus*, the lethal effects are reversed by the addition of Fe in several forms. As further evidence of its Fe requirement, *V. vulnificus* produces both the hydroxamate and phenolate classes of siderophores when grown in an Fe-limiting environment (20). The role of heme Fe as a source of metabolic nutrition for *V. vulnificus* has not been examined.

V. vulnificus has been shown to produce hemolysins (D. E. Johnson and F. M. Calia, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B60, p. 24); therefore, septicemia after wound infections may be accompanied by an increase in free plasma Hb and nutritional Fe. Eaton et al. (7), however, suggest that Hb-driven septicemia should be rare due to the complexing of serum haptoglobin (Hp) to Hb. Hp, a naturally occurring Hb-binding protein, functions to prevent the loss of Hb during intravascular hemolysis by preventing passage of free Hb through the glomeruli (2, 10, 16). The stable complex of Hp and Hb is rapidly cleared by the reticuloendothelial system (8, 9). Eaton et al. (7) observed that the adjuvant effect of free Hb on the lethality of a clinical isolate of *E. coli* injected intraperitoneally is blocked when enough purified Hp is added to bind all Hb. This study suggests that Hp renders the Fe of Hb unavailable for incorporation into bacterial Fe-binding proteins, thereby exerting a bacteriostatic effect.

We report here studies (i) to determine the effect of Hb upon the lethality of *V. vulnificus*, (ii) to characterize the ability of *V. vulnificus* to acquire nutritional Fe from Hb and other heme-containing molecules, and (iii) to determine whether *V. vulnificus* is blocked from extracting Fe from the Hp-Hb complex normally found in serum.

MATERIALS AND METHODS

Animals. ICR mice used in this study were originally obtained from Flow Laboratories (Rockville, Md.) and maintained in the animal facilities at the University of North Carolina at Charlotte. Both males and females were used, ranging in age from 6 to 8 weeks.

Glassware and solutions. Except for disposable glass pipettes (Corning Glass Works, Corning, N.Y.), which have been found to contribute negligible amounts of contaminat-

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ing Fe (14), all glassware used in the in vitro studies were washed overnight in 6N HCl and rinsed 10 times with glass-distilled water. All solutions used in the in vitro studies were prepared with glass-distilled water.

Preparation of bacterial cells. Before use, *V. vulnificus* (strain C7184 obtained from the Centers for Disease Control, Atlanta, Ga.) was cultivated for 3 to 4 h in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) on a rotary shaker at 37°C at ca. 200 rpm. The inoculum size for all intraperitoneal (ip) injections was standardized by determining optical density of the culture on a Bausch & Lomb Spectronic 20 spectrophotometer and confirmed for each experiment by total viable cell counts, in duplicate, on brain heart infusion agar. An inoculum size of ca. 10^3 bacteria (50% lethal dose, 10^6; see reference 22) suspended in 0.1 ml of phosphate-buffered saline (PBS) was used for all ip injections.

For in vitro experiments, *V. vulnificus* C7184 or *E. coli* K-12 was obtained from the early stationary growth phase in brain heart infusion broth and washed three times in PBS. The inoculum size was determined as described above. Approximately 10^4 cells suspended in 0.1 ml of PBS were used for inoculation into defined media or sera.

Preparation of deferrated medium. Synbase, a low-Fe medium, was prepared as described by Payne and Finkelstein (18), except that dextrose replaced sucrose. To prepare a deferrated medium, contaminating Fe was removed from Synbase by using the ion-exchange resin Chelex-100 (Bio-Rad Laboratories, Rockville Centre, N.Y.) as described by Mickelsen and Sparling (14). The resin was added to the medium and mixed gently overnight at 4°C. The medium was removed from the resin and treated two additional times with fresh Chelex-100. The deferrated medium was supplemented with 100 μM $CaCl_2$–1.0 mM $MgCl_2$, and the pH was adjusted to 7.2. The medium was then sterilized by passage through a 0.20-μm-pore-size membrane filter (Nuclepore Corp.).

Preparation of human serum for bacterial culture. Human blood was drawn aseptically from volunteers and allowed to clot at room temperature for 1 h. Serum was obtained by centrifugation of the clotted blood for 10 min at 8,000 × *g* and heat inactivated by incubation at 57°C for 30 min. Various amounts of Hb (ranging from 1.0 to 3.0 mg) were added to 1-ml portions of serum and assayed for free Hb by polyacrylamide gel electrophoresis to determine the Hb-binding capacity of the serum.

Preparation of heme-containing molecules. Hb, methemoglobin (metHb), and myoglobin (Myb) in lyophilized form were obtained commercially (Sigma Chemical Co., St. Louis, Mo.), or fresh Hb was prepared from erythrocyte lysates. These molecules were dissolved in PBS in Fe-equivalent amounts for use in the various experiments. Hemin (Sigma) was suspended in PBS and then converted to hematin (He) by adding 1.0 N NaOH until solubility was obtained at ca. pH 8.0.

Induction of hemoglobinemia. Phenylhydrazine (PHZ) was injected ip (100 mg/kg of body weight) to induce levels of free Hb in the plasma of mice. Plasma was obtained from mice by retro-orbital puncture and assayed for Hb levels spectrophotometrically (6). Hb was converted to cyanomethemoglobin by adding 0.9 ml of Drabkins solution to 0.1 ml of plasma. Absorbances of the Hb solutions were read at 540 nm on a Beckman model 25 spectrophotometer. The concentrations of Hb in the plasmas were determined from a standard curve constructed from Hb standards treated as described. To differentiate between the total concentration of Hb in the plasma and the concentration of free Hb (not bound to protein), the plasma samples were assayed for their percentages of free Hb and Hb-Hp by polyacrylamide gel electrophoresis. The relative percentages of each was determined by comparison of the areas under the defined peaks of each sample after scanning of the gels with an integrated densitometer.

RESULTS

To assess the role of Hb as an adjuvant for the pathogenicity of *V. vulnificus*, the induced peritonitis model was used. In this study, various amounts of FAC were administered to mice, delivering from 1.73 to 6.92 μg of Fe per injection. A second group of mice received Hb in doses that were Fe equivalent to those of the FAC injections. Both groups received concurrent ip injections of a normally sublethal number (ca. 10^3) of *V. vulnificus* cells. Control mice received inocula of bacteria, Hb, or FAC only. At 24 h postinjection, the percent lethality for mice in each Fe dosage group was calculated. Control animals exhibited no deaths. FAC enhanced the lethality of *V. vulnificus* when present concurrently in the peritoneal cavities of mice, with a direct correlation existing between the dose of Fe and percent lethality (Fig. 1). The lethality curve for Hb-injected mice exhibited the same characteristics.

Heme-containing molecules other than Hb were tested for their ability to enhance the lethality of *V. vulnificus*. Various amounts of either Myb, metHb, or He were injected ip into experimental animals concurrently with ca. 10^3 bacterial

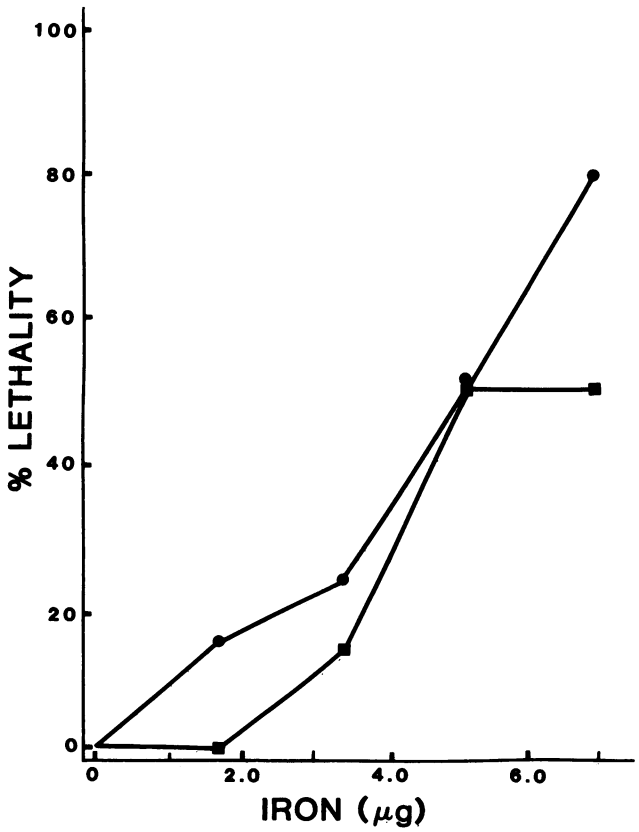

FIG. 1. Lethality curves for mice with induced peritonitis. Each point represents the percentage of deaths in a group of 10 animals which received challenges of *V. vulnificus* (10^3 cells) concurrently with Fe-equivalent amounts of Hb (circles) or FAC (squares).

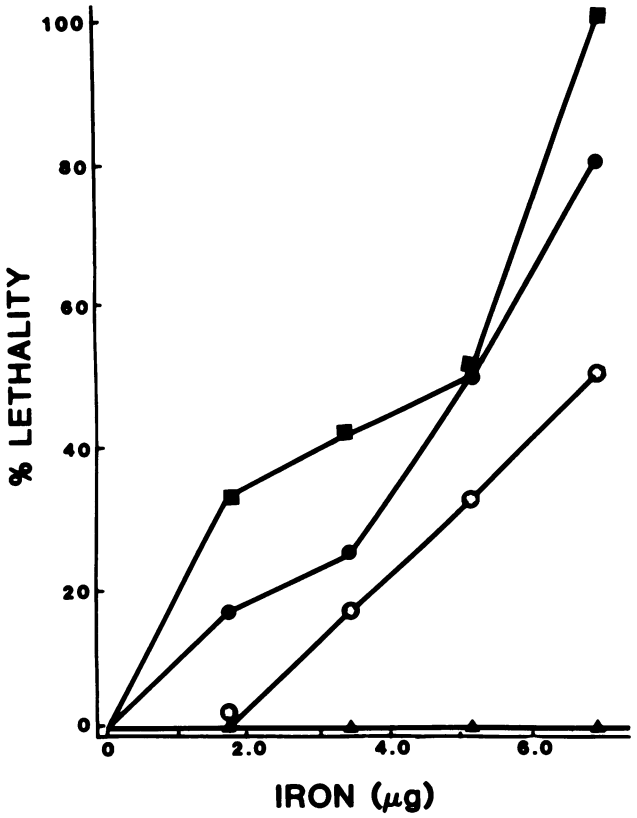

FIG. 2. Lethality curves derived from the induced peritonitis model. Each point represents the percentage of deaths in a group of 10 animals which received challenges of *V. vulnificus* (10^3 cells) concurrently with Fe-equivalent amounts of He (squares), Hb (closed circles), metHb (open circles), or Myb (triangles).

cells. At 24 h postinjection, the percent lethality of mice for each Fe dose of each molecule was determined. No deaths were recorded in control groups, which received either bacteria or the largest dose of the various heme-containing molecules. The lethality induced by the *V. vulnificus* challenge correlated directly with increasing amounts of metHb and He (Fig. 2). These two curves are similar to the Hb curve, suggesting similar mechanisms of enhancement of lethality. Myb, however, exerted no adjuvant effect in the range of Fe depicted in Fig. 2. Attempts to induce lethality with higher doses of Myb (containing up to 14 μg of Fe) also failed.

In addition to the induced peritonitis model, PHZ-induced hemoglobinemia was used to study further the adjuvant effect of Hb upon the pathogenicity of *V. vulnificus*. PHZ induced lysis of erythrocytes, resulting in free Hb in the plasma for up to 96 h postinjection. To correlate the degree of hemoglobinemia with the pathogenicity of *V. vulnificus*, mice were bled at intervals after PHZ injections, and the levels of total and free Hb (Hb not conjugated with Hp) were determined. After the bacterial challenge, mice were monitored, and the times of death were recorded. The lethality induced by the *V. vulnificus* challenge was 100% at 12, 24, and 48 h after PHZ injection (Fig. 3). At these same times, both total and free plasma Hb levels were elevated above controls. At 96 h after PHZ injection, however, the concentration of total and free Hb had fallen to normal levels, and this decrease was accompanied by a drop in lethality from 100 to 45%.

To assess the role of heme Fe as a source of nutritional Fe for *V. vulnificus*, an in vitro system with a deferrated medium was used. The deferrated medium was supplemented with either Hb, Myb, or FAC as the source of Fe. Medium not supplemented with Fe served as a control. An inoculum size of ca. 10^4 cells was added to each test system. At various time intervals, total viable cell counts were performed from each culture. Data in Fig. 4 indicate that the deferrated medium alone supported no growth, with the total viable cell count dropping to 10^2 within 8 h, whereas media supplemented with Hb, Myb, or FAC supported significant growth of this organism.

To determine whether *V. vulnificus* is inhibited from extracting Fe from the Hp-Hb complex normally found in serum, an in vitro study with human serum as a growth medium was conducted. A duplicate study with *E. coli* was also run. Serum alone did not support the growth of either *E. coli* or *V. vulnificus* (Fig. 5). Serum supplemented with Hb above the Hb-binding capacity of the serum (3 mg/ml), however, supported the growth of both. When the serum was supplemented with Hb below the Hb-binding capacity of the serum (1 mg/ml), the growth of *E. coli* was not supported, although the growth of *V. vulnificus* was supported. In cultures with the addition of FAC in an amount that was Fe equivalent to 1.0 mg/ml of Hb, the growth of both was supported, indicating that the limiting factor for *E. coli* was not the amount of Fe but its accessibility. Therefore, unlike *E. coli*, *V. vulnificus* appears to be able to overcome the blocking effect of Hp and acquire Fe from the Hp-Hb complex.

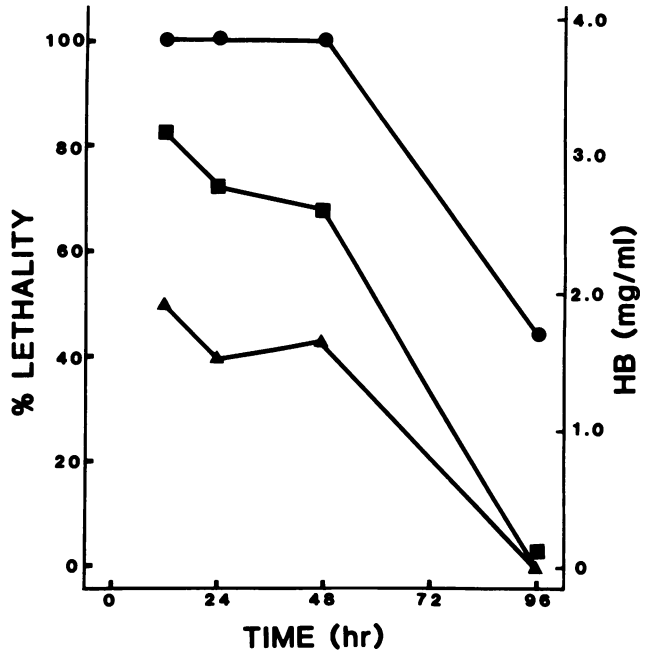

FIG. 3. Relationship between lethality of *V. vulnificus* challenge and degree of PHZ-induced hemoglobinemia. Total plasma Hb (squares), free plasma Hb (triangles), and percent lethality (circles) are plotted against time (hours after PHZ injections). Each point represents the average value for groups of 10 mice. Hb values for a group of control mice (receiving no PHZ) were determined and subtracted from these averages to adjust for incidental lysis.

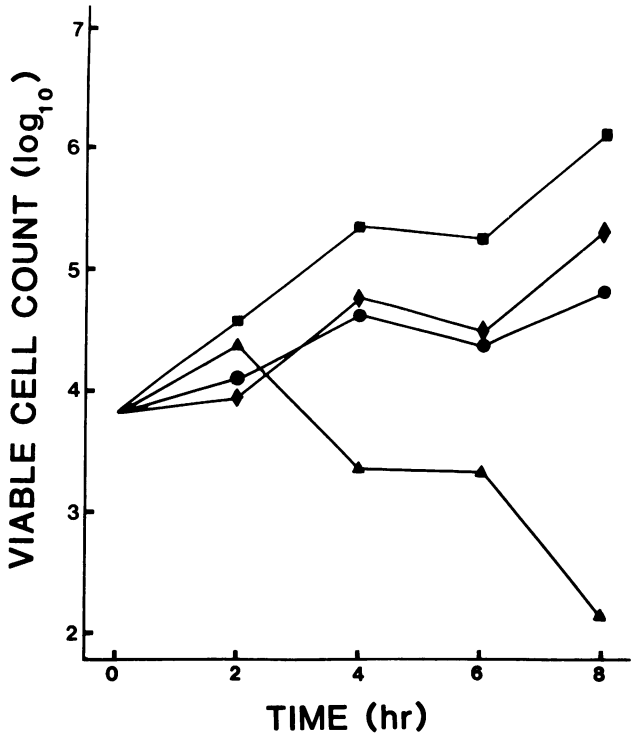

FIG. 4. Growth curves for *V. vulnificus* in deferrated medium supplemented with no Fe source (triangles), FAC (squares), Hb (circles), or Myb (diamonds). Lyophilized human Hb was dissolved directly in the medium at 1 mg/ml. FAC and Myb were added in Fe-equivalent amounts.

DISCUSSION

For many years it has been recognized that when Hb and bacteria coincide in a wound, life-threatening infections often occur. In recent years, it has been suggested that the Fe moiety of the Hb molecule is responsible for this increased pathogenicity. Most studies on this adjuvant effect of heme Fe have been conducted with enteric bacteria, and little information is available for other pathogenic species, such as the vibrios. A variety of models have been used to study the effect of heme-containing molecules on the pathogenicity of bacteria. As previously reported (22), we found the nonheme iron source (FAC) to enhance the lethality of *V. vulnificus* when injected ip (Fig. 1). Data from the induced peritonitis model also demonstrated that Hb, metHb, and He increased the pathogenicity of infections by *V. vulnificus* (Fig. 1 and 2). Although these data alone do not confirm that the Fe of heme is responsible for this increase in pathogenicity, previous studies (21) have shown that when globin is injected concurrently with bacteria, no deaths occur. In addition, we have observed that albumin and gamma globulin exhibit no adjuvant activity (data not shown). However, He (the Fe-containing porphyrin ring structure of Hb) exerts an adjuvant effect on the pathogenicity of *V. vulnificus* (Fig. 2). These observations, along with the similarity of the Hb, metHb, and He lethality curves with the lethality curve for nonheme Fe (FAC), suggest strongly that the Fe moiety is responsible for the enhanced pathogenicity.

To examine further whether the Fe of Hb was the factor contributing to the increase in pathogenicity of *V. vulnificus*, an in vitro study was conducted. When bacteria were inoculated into a deferrated medium which contained Hb as the only source of Fe, growth of the organism was supported, and the growth curve was very similar to that supported by FAC (Fig. 4). These observations support the concept that the Fe moiety of Hb serves as a nutritional factor for growth and, thereby, enhances pathogenicity.

In contrast to the adjuvant effect of Hb, metHb, and He, ip injections of Myb in Fe-equivalent doses did not increase the pathogenicity of sublethal inocula of *V. vulnificus* (Fig. 2). These initial studies suggest that *V. vulnificus* is incapable of extracting the heme Fe from Myb. However, in vitro experiments demonstrated that the bacterium is capable of growth in deferrated medium with Myb as the only source of Fe (Fig. 4). The factors contributing to the inaccessibility of Fe from Myb in vivo can only be speculated on. It is possible that the clearance of Myb from the peritoneal cavity is more rapid than the clearance of other heme-containing molecules, thereby not allowing the utilization of its Fe compo-

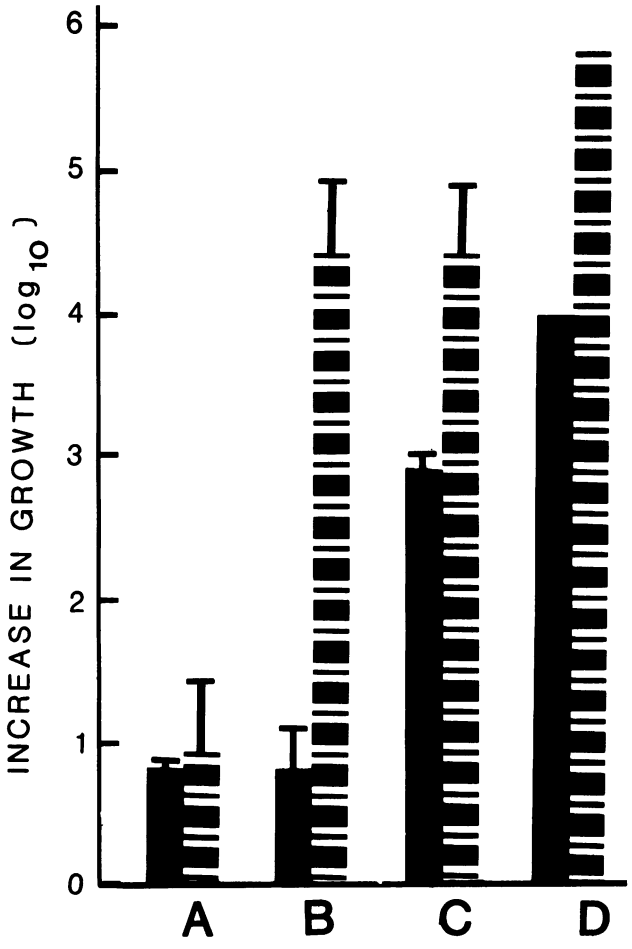

FIG. 5. Eight-hour growth for *E. coli* (closed bars) and *V. vulnificus* (hatched bars) in human serum. A, Serum only; B, serum supplemented with Hb (1 mg/ml); C, serum supplemented with Hb (3 mg/ml); and D, serum supplemented with FAC (Fe equivalent to 1 mg of Hb). Each bar represents the increase in bacterial growth above the original inoculum (ca. 10^4 cells) and is the average of three experiments with the range indicated. The FAC-supplemented cultures (D) were included in only one experiment. The Hb in B was completely complexed with Hp, as monitored by acrylamide gel electrophoresis, although ca. 50% of the Hb in C existed as free Hb.

nent. It was observed that the adjuvant effect of Hb on the pathogenicity of *V. vulnificus* was lost 24 h after its injection (data not shown), presumably due to clearance from the peritoneal cavity by cells of the reticuloendothelial system.

We have used PHZ-induced hemoglobinemia in mice to study further the adjuvant effect of Hb upon the pathogenicity of *V. vulnificus*. Mice exhibiting levels of free plasma Hb above control levels showed increased susceptibility to *V. vulnificus* infections (Fig. 3). However, at 96 h after PHZ injection, when free Hb levels had returned to normal, the percent lethality of the normally sublethal challenge of bacteria was still 45%, suggesting that other factors resulting from PHZ injections may also contribute to increased sensitivity to infection. One such factor may be the release of free iron, thereby increasing the degree of transferrin saturation. Therefore, although the data from this model, in conjunction with other data presented, support the concept of Hb-enhanced bacterial pathogenicity, caution must be taken with interpretations of these data.

The role of Hb in the natural course of infections produced by *V. vulnificus* can only be surmised. This bacterium has been shown to produce hemolysins (D. E. Johnson and F. M. Calia, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B60, p. 24). Therefore, septicemia may be accompanied by an increase in free plasma Hb and nutritional Fe. Eaton et al. (7), however, suggest that Hb-driven septicemia should be rare. These investigators, using both the induced peritonitis model and in vitro growth in Fe-free medium, have shown that if enough Hp is introduced into each system to completely bind all added Hb, the normal adjuvant effect of Hb is reversed for *E. coli*. The binding of Hp to Hb would thus eliminate a major reservoir of Fe for most pathogens and may be added to the list of possible iron-withholding activities exhibited by infected hosts. Our data (Fig. 5) indicate, however, that *V. vulnificus* has the ability to overcome this putative bacteriostatic effect and acquire Fe from the Hp-Hb complex. This ability may explain, in part, the relatively high virulence of this species.

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