Ability of Vibrio vulnificus to Obtain Iron from Hemoglobin-Haptoglobin Complexes

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It has been suggested that the normal serum protein, haptoglobin (Hp), serves a bacteriostatic role by binding free hemoglobin (Hm), thus making heme iron unavailable for bacterial growth. Previous studies showed that, unlike Escherichia coli, Vibrio vulnificus was able to overcome this Hp-blocking effect. We report here a study on the iron-withholding property of the three major human Hp phenotypes, Hp 1, 2, and 2-1. Results of experiments with human serum showed that V. vulnificus C7184 was able to obtain iron from Hm bound to Hp types 1 and 2, but not that bound to Hp 2-1. E. coli 2395-80, on the other hand, was unable to overcome the blocking effect of any Hp phenotype. Using purified Hp 1, we also demonstrated that, although V. vulnificus was unable to grow in a deferrated medium without an additional iron source, it was able to grow with the addition of the Hm-Hp complex.

Bacteria, like most other organisms, require iron for cellular functions. The majority of iron in humans is bound to iron-binding proteins, such as transferrin and lactoferrin, or sequestered in ferritin, hemosiderin, myoglobin, and hemoglobin (3, 12). The presence of these proteins and complexes results in a free iron concentration in serum of 10⁻¹⁰ M (7). Since most bacteria require approximately 10⁻⁶ M iron for growth, the ability of an organism to acquire iron from the host may contribute to its pathogenicity (6, 18).

Vibrio vulnificus is an opportunistic marine pathogen capable of causing fatal septicemias and wound infections in humans. Wright et al. (20) showed that iron overload in mice reduced the 50% lethal dose of V. vulnificus from 10⁶ cells to a single cell. Helms et al. (8) further demonstrated in vitro and in vivo that V. vulnificus is able to utilize various heme-containing compounds, including hemoglobin (Hm), as an iron source. Free Hm has repeatedly been shown to have a synergistic effect on the lethality of intraperitoneal or subcutaneous inocula of Escherichia coli and other gram-negative bacteria (2, 10). It has also been established that the iron in Hm is the component responsible for the enhancement of virulence (5). Eaton et al. (5) had earlier suggested that Hm-driven bacterial growth may be rare in vivo because of the presence of haptoglobin (Hp), a normal serum protein that binds specifically and irreversibly to Hm (4). Those authors suggested that this Hp-Hm complex rendered the heme iron of Hm unavailable for a clinical strain of E. coli. They suggested further that Hp, with its iron-withholding property, may serve a bacteriostatic role. Helms et al. (8) confirmed this finding with E. coli but found that V. vulnificus was able to overcome the Hp-blocking effect.

Three major human Hp phenotypes, Hp 1, Hp 2, and Hp 2-1, are known to exist (15, 17). Hp 1 is a monomeric protein, whereas Hp 2 and Hp 2-1 both exist in the polymorphic form. Because the molecular weights of Hp 1, Hp 2, and Hp 2-1 are quite different, it seems that the binding of Hp to each Hp type would give rise to very different complexes. In the present study, the three human Hp phenotypes were compared for their efficiency in withholding Hm iron from V. vulnificus and E. coli.

Blood was drawn aseptically from human volunteers and centrifuged at 8,000 × g, and the serum was removed. The serum samples were heat inactivated at 57°C for 30 min, filter sterilized, and stored at 4°C until used. The phenotype of each serum sample was determined by using polyacrylamide gel electrophoresis and staining with dimethoxybenzidine as described by Sutton (17). Serum samples from at least three different donors with similar Hp phenotypes were pooled for use in these studies. Preparations containing various amounts of human Hm (Sigma Chemical Co., St. Louis, Mo.; dialyzed and lyophilized) suspended in Tris-glycine buffer were added to the sera and assayed by polyacrylamide gel electrophoresis to determine the Hm-binding capacity of the three pooled samples. The binding capacity was confirmed by using G-100 (Sephadex) gel filtration column chromatography.

V. vulnificus C7184 and an enteropathogenic strain of E. coli, 2395-80 (obtained from the Center for Vaccine Development, Baltimore, Md.), were grown for 6 to 8 h in heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C. The cells were then harvested and washed three times with sterile phosphate-buffered saline. The bacterial cells were then added to preparations containing serum or serum with Hm added below the saturation level of Hp. In serum of Hp phenotypes 1 and 2, V. vulnificus was consistently killed in the presence of serum alone but was able to reverse this killing in the presence of Hm, even at levels at which all the added Hm was bound to Hp (Table 1). The growth of E. coli in serum with no free Hm was not significantly different from that in serum alone (Table 1). Unlike that of V. vulnificus, its growth was enhanced only in preparations containing free Hm (data not shown).

In contrast, whereas V. vulnificus was again killed in the Hp 2-1 serum, the addition of Hm at a level at which it would all be bound to Hp did not reverse the killing effect of serum (Table 1). However, the killing was reversed with the addition of excess Hm (data not shown). This suggests that, like E. coli, V. vulnificus was not able to overcome the blocking effect of Hp 2-1.

To determine whether the degree of saturation of Hp with


Hm affected the ability of the organisms to acquire heme iron, Hm in amounts from 0 to 3.0 mg was added to 1-ml preparations of serum of Hp 1. Bacteria were added and incubated for 12 h, and total viable counts were determined.

The addition of Hm at levels below the binding capacity of the Hp in the serum reversed the killing effect of serum for V. vulnificus (Fig. 1). In fact, growth appeared to be proportional to the amount of Hm in the Hm-Hp complex.

To further demonstrate that the growth enhancement was due to the ability of V. vulnificus to acquire Hm from the Hm-Hp complex, we examined the growth of this organism in a deferrated, defined medium. Synbase was deferrated with Chelex-100 as previously described (16). Hm was added to purified Hp 1 (Calbiochem-Behring, San Diego, Calif.) to obtain levels of saturation above and below the binding capacity. These Hm-Hp complexes were added to deferrated synbase to obtain a final Hp concentration of 1 mg/ml and inoculated with 10^8 bacterial cells. Samples were incubated at 37°C for 12 h, and total viable counts were determined. Growth in control tubes containing the deferrated medium alone and in a medium with Hp was also determined.

This deferrated, defined medium failed to support growth unless Hm was added (Fig. 2). As was observed with serum, V. vulnificus was able to grow in the defined medium with Hm even when all was bound to Hp. The addition of Hp alone failed to enhance growth (data not shown).

The ability of an organism to override the blocking effect of Hp may be a potentially strong virulence factor. Approximately 57.6% of the iron in the body is contained in Hm (19). Upon lysis of erythrocytes, Hp forms a complex with Hm, preventing passage of Hm through the glomeruli (15). If an organism were capable of acquiring iron from this complex, its survival and growth in the host could be greatly enhanced. In our study, E. coli was incapable of obtaining iron from the Hm-Hp complex regardless of the Hp phenotype. In contrast, V. vulnificus could obtain iron from Hm bound to Hp 1 and Hp 2. Hp 2-1, however, seemed to efficiently block utilization of iron from the bound Hm. Since the exact mechanism by which V. vulnificus is able to obtain iron from the Hm-Hp complex has not been determined, it is unclear why V. vulnificus is able to override the Hp 1 and 2 blocking effect but not that of Hp 2-1. Although there are reports describing the nature of the binding of Hm to Hp 1 (1, 4, 13), there is little information available regarding the binding to the polymeric types. Previous studies (9, 11) have shown that some bacteria have binding sites for Hp, but these studies were done by using only Hp 1 or Hp 2 from pooled sera. Kohler and Prokop (9) reported a difference among the three phenotypes and their ability to cause agglutination of Streptococcus pyogenes, suggesting a difference in binding of Hp to the bacterial cell. If it is necessary for the bacterial cell to bind Hp before Hm iron is available, differences in binding could explain the differences between E. coli and V. vulnificus as well as results of our phenotypic studies. Since proteases from Staphylococcus aureus have been shown to cleave the Hp molecule at numerous sites (13), it is possible that variations in protease activity also explain these differences. V. vulnificus has been shown to produce a variety of enzymes with proteolytic activity (14), and one or more of these may degrade the complex, resulting in free Hm for utilization by the bacterial cell.

Our data (Table 1) confirm our earlier study (8) showing that, whereas E. coli is not able to override the effect of Hp, V. vulnificus is able to efficiently remove Hm even at levels at which it is all bound. That the enhanced growth is due to Hp is evidenced by the increase in cell number that is observed as the amount of Hp increases (Fig. 1). The present study suggests that V. vulnificus has an extremely effective mechanism for using Hp.

The fact that V. vulnificus was unable to grow in deferrated medium unless iron or Hp was added provides strong evidence that this organism has an effective mechanism for overriding the bacteriostatic effect of Hp. This potential virulence factor, along with the efficient use of Hp by this

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**TABLE 1. Effect of Hp phenotype on bacterial growth**

<table>
<thead>
<tr>
<th>Hp phenotype</th>
<th>V. vulnificus</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.7</td>
<td>-0.02</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>0.03</td>
</tr>
<tr>
<td>2-1</td>
<td>-0.01</td>
<td>-0.01</td>
</tr>
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*Log increase from growth in human serum without Hp addition. Values are averages of a minimum of three replica experiments.*

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**FIG. 1.** Twelve-hour growth of E. coli and V. vulnificus in Hp 1 serum supplemented with various amounts of Hm. The Hm-binding capacity (HbBC) of the serum is designated and lies between 1.25 and 1.50 mg of Hm/ml of serum. The bars represent the standard error of the mean of values obtained in up to as many as three runs. Hb, Hemoglobin.

**FIG. 2.** Growth enhancement of V. vulnificus with the addition of Hm or Hp-Hm to a deferrated medium. Symbols indicate growth: ■, in deferrated medium; ○, with Hp; ●, with Hp-Hm complex.
organism, may help account for its ability to become invasive.

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LITERATURE CITED