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In Vivo Function of Hemolysin in the Nephropathogenicity of *Escherichia coli*

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The role of hemolysin in the nephropathogenicity of *Escherichia coli* was studied in a hematogenous pyelonephritis model in mice. The nephropathogenicity of a nonhemolytic, avirulent *E. coli* strain was increased by simultaneous injection with its hemolytic, nephropathogenic parent. This helper mechanism could be attributed to hemolysin, since the simultaneous injection of partially purified hemolysin gave a similar enhancement of nephropathogenicity. Intraperitoneal injection of hemoglobin or iron sulfate before intravenous challenge with this avirulent strain also led to increased virulence. The nephropathogenicity-enhancing effect of hemolysin is therefore supposed to depend on increasing the level of available iron in the host. Under conditions of plentiful iron, hemolysin production was repressed, as shown by in vitro growth experiments in the presence of exogenous iron. These results suggest that the production of hemolysin is regulated by feedback inhibition.

With the use of a hematogenous pyelonephritis model in mice, *Escherichia coli* strains can be placed in one of three virulence groups (19): (i) avirulent strains, (ii) nephropathogenic strains, and (iii) strains with a general virulence. We have previously reported that the hemolytic strain CW22 behaves like a nephropathogenic strain in this model (21). The presence of a 42-megadalton plasmid that codes for hemolysin production was found to be responsible for the nephropathogenicity of this strain.

In an attempt to determine whether hemolysin itself or some other plasmid-encoded property contributes to virulence the nephropathogenicity was analyzed of strains which carried plasmid derivatives obtained after transposon insertions. Insertions that affected the hemolysin production were found to reduce the virulence for mouse kidneys (20a), suggesting that hemolysin is involved in the virulence of nephropathogenic *E. coli* strains.

To investigate the role of hemolysin in the virulence of *E. coli* for mice, reconstitution experiments were carried out that could lead to a better understanding of the function of hemolysin in vivo.

The lethality of *E. coli* and hemoglobin together is well recognized among clinicians and can be abolished by the hemoglobin-binding protein haptoglobin (9). The virulence-enhancing effect of hemoglobin is also observed with hematin, heme, or iron salts (5). Furthermore, the enhancement of virulence in experimental infections by simultaneous injection with iron has been reported for a variety of bacterial species (for a recent review, see reference 3). This suggests that it is the iron moiety of hemoglobin that is responsible for the enhanced virulence.

The purpose of this paper is to establish whether hemolysin may act as a virulence factor for nephropathogenic *E. coli* strains by the liberation of hemoglobin, resulting in increased levels of iron compounds that stimulate bacterial growth in the animal.

MATERIALS AND METHODS

Bacteria. The hemolytic *E. coli* strain used in this study, CW22 (O139:K82:H1), is a mutant of the natural isolate P673 that has retained nephropathogenicity for mice (21). The nonhemolytic strain CW101 is a mutant of CW22, obtained after Tn*5* transposon mutagenesis, that has lost its virulence for mice (20a).

Preparation of partially purified hemolysin. The strains CW22 and CW101 were grown overnight in alkaline meat extract broth containing 0.2% glucose (18). These cultures were diluted 10-fold with fresh medium and grown for approximately 2.5 h to yield late log phase cells. After removal of the cells by centrifugation, the supernatant was sterilized by passage through a 0.45-μm filter. Hemolysin was partially purified by precipitation with 28% (wt/vol) ammonium sulfate and was redissolved in half the original volume of one-fourth strength Ringers solution.

Virulence test. Virulence was tested in an experimental mouse model (19). Bacteria were grown to late

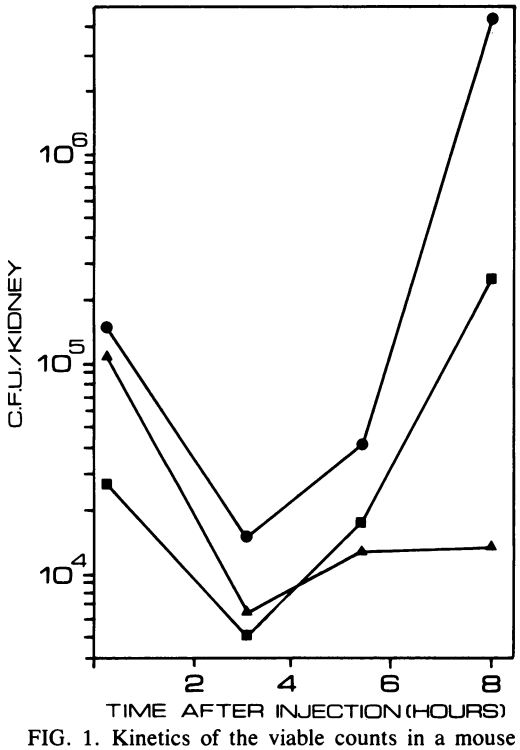

FIG. 1. Kinetics of the viable counts in a mouse kidney after intravenous injection. Symbols: ●, total viable count after injection with 1×10^8 hemolytic and 1×10^8 nonhemolytic bacteria; ■, viable count of the nonhemolytic bacteria from this infection mixture; ▲, total viable count after injection of 2×10^8 nonhemolytic bacteria. At time zero, 10 mice were injected with 20×10^8 bacteria each, and at the times indicated, the viable counts in the kidneys of two mice were determined. Symbols represent the average of two experiments.

log phase to yield 10^9 cells per ml. Then 2.5×10^8 viable cells were injected intravenously into 8-week-old female Swiss-Random mice (approximate weight, 25 g), and the number of viable counts in each kidney was determined during the 8 h after injection. Avirulent strains showed a rapid and progressive decline in kidney viable counts. Group II strains showed an initial decrease in kidney viable counts, followed by an increase 3 to 4 h after injection, and the mice were generally killed within 4 days. These strains were designated nephropathogenic, since the rise in viable counts was not observed in other organs. Finally, group III strains or generally virulent strains showed high counts in all organs, and the mice usually died within 8 h.

Hemoglobinuria, as judged by the brownish appearance of the bladder content, was observed only in animals that were injected with hemolysin or hemolytic bacteria. Hemoglobinuria was associated with a significant increase of hemoglobin absorbance in cell-free blood samples from these animals due to intravascular hemolysis. Since mouse blood is very liable to hemolyze spontaneously, in later experiments we used hemoglobinuria as a sign of intravascular hemolysis.

Growth stimulation by iron compounds. Blood agar base no. 2 (Oxoid, Basingstoke, England) plates, containing a 100 μM concentration of the iron-chelating compound 2,2-dipyridyl (BDH, England), were seeded with a bacterial suspension. Subsequently, small pieces of filter paper, soaked in 10 mM ferric chloride, ferric citrate, or ferrous sulfate, were placed onto the plates, and bacterial growth was monitored over a 72-h period.

Measurement of hemolytic activity. Strain CW22 was grown in alkaline meat extract broth containing various concentrations of $FeCl_3$. Cultures grown overnight were diluted 10-fold in similar fresh medium and were grown for 2.5 h. The hemolytic activity was determined as described previously (20, 20b). Briefly, serial dilutions of the supernatant were made after neutralization. These dilutions were incubated with an equal volume of washed sheep erythrocytes (2%) for 2 h at 37°C. After removal of unlysed erythrocytes by centrifugation, the amount of hemoglobin in the supernatant was measured spectrophotometrically (λ = 540 nm). The hemolytic titer was defined as the highest dilution that lysed more than 25% of the erythrocytes.

RESULTS

Enhancement of the nephropathogenicity of CW101 by simultaneous injection with CW22. We previously showed that the hemolytic strain CW22 behaved like a nephropathogenic strain in the experimental mouse model (21). When the ability to produce hemolysin was lost by curing the strain for its hemolysin plasmid, the strain became avirulent. Similar effects were found when hemolysin production was lost by insertional inactivation with transposon Tn*5* in the plasmid gene(s) responsible for hemolysin production (20a).

In an attempt to determine the reason for this decrease in virulence, we injected mice with a mixture of the hemolytic strain CW22 and the nonhemolytic strain CW101. To determine whether the bacteria recovered from the kidneys still consisted of a mixture of CW22 and CW101, kidney homogenates were plated on selective media that discriminated between both strains (e.g., hemolysin production or resistance to kanamycin). Since identification of hemolytic and nonhemolytic colonies is only accurate when low numbers of bacteria are plated, the majority of experiments were performed with plates containing 20 μg of kanamycin.

When a mixture of equal numbers of CW22 and CW101 was injected into mice, the kinetics of the total kidney viable counts resembled those found with nephropathogenic strains (Fig. 1). To determine which of the constituents of the infection mixture was responsible for this increase, kidney homogenates were also plated on selective media. These platings showed that the increase in total kidney viable counts was caused by an increase of both the virulent and the avirulent strain (Fig. 1). In the presence of

the hemolytic strain CW22, the number of viable CW101 bacteria in the kidney increased, in contrast to the injection of only nonhemolytic CW101 bacteria (Fig. 1).

Enhancement of the nephropathogenicity of CW101 by hemolysin. From 3 h after infection with hemolytic strains, mice showed hemoglobinuria, which they did not after infection with nonhemolytic strains. After infection with the mixture of CW22 and CW101, hemoglobinuria was also observed. We suspected hemoglobinuria to be the result of intravascular hemolysis. In vivo activity of hemolysin was demonstrated by a significant increase of cell-free hemoglobin in blood samples from mice infected with the hemolytic strain CW22.

To determine whether the virulence-enhancing effect, observed with mixtures of hemolytic and nonhemolytic bacteria, was caused by hemolysin, mice were injected with strain CW101 suspended in 0.5 ml of partially purified hemolysin from CW22. Again, intravascular hemolysis and hemoglobinuria were observed, and between 3 and 8 h after infection, kidney viable counts increased (Fig. 2). To exclude the possibility that other constituents from this hemolysin preparation were responsible for the increase in the virulence of CW101, mice were injected with CW101 suspended in identically processed supernatant from strain CW101. In these control mice, no sign of hemoglobinuria could be observed. Furthermore, this hemolysin-free supernatant did not exert a virulence-enhancing effect on CW101 (Fig. 2).

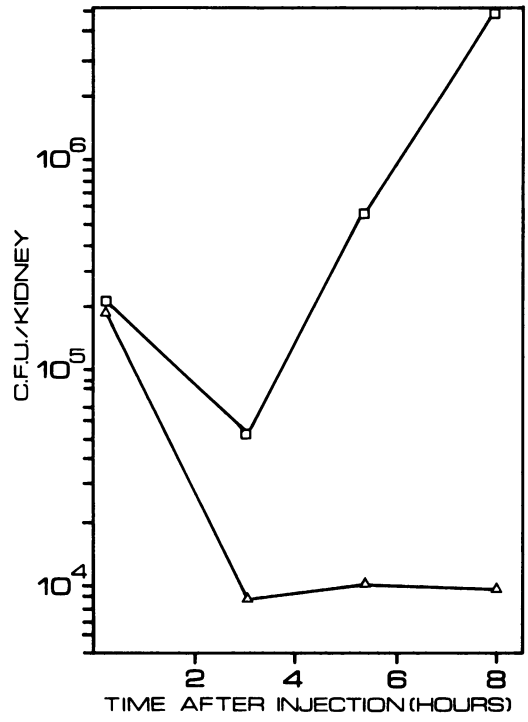

FIG. 2. Kinetics of the total kidney viable count of the nonhemolytic strain CW101 after simultaneous injection of partially purified supernatants. Symbols: □, supernatant from the hemolytic strain CW22; △, supernatant from the nonhemolytic strain CW101. Each symbol represents the mean viable count in both kidneys of two mice. According to the Student *t* test, the differences between both patterns are significant ($P < 0.01$).

Enhancement of the nephropathogenicity of CW101 by hemoglobin and $FeSO_4$. The intravascular hemolysis that was observed in mice injected with either hemolytic bacteria or partially purified hemolysin suggested that it could be the erythrocyte content, in particular hemoglobin, that enhanced the virulence of CW101. Therefore, we injected mice intraperitoneally with 1.4 μM hemoglobin and, 30 min later, intravenously with 2×10^8 CW101 bacteria. This combination led to a rise in kidney viable counts between 3 and 8 h after infection (Fig. 3).

The increased virulence of strain CW101 observed in this experiment could be caused by hemoglobin-mediated growth stimulation. To test this, several *E. coli* strains were grown under iron-limiting conditions, using the chelating compound 2,2-dipyridyl as described above. No growth occurred on these plates, showing that the concentration of dipyridyl was sufficient to inhibit growth. On the other hand, disks containing 4 mg of hemoglobin per ml permitted bacterial growth, as did disks with various iron salts.

Since it has been shown that the iron moiety of hemoglobin causes the enhancement of general virulence of *E. coli* (5), we infected mice with strain CW101 after intraperitoneal injection of 1.4 μM $FeSO_4$. Although the decline in kidney viable counts in the first 3 h after injection was not prominent, viable counts increased thereafter (Fig. 3), and the remaining mice died within 48 h, indicating restoration of the nephropathogenicity of the nonhemolytic mutant by iron.

Inhibition of hemolysin production by iron. From the results mentioned in the previous sections, we hypothesized that hemolysin exhibits its virulence-enhancing effect by increasing the amount of available iron. To substantiate this, the role of iron in the production of hemolysin was investigated. In these experiments, *E. coli* CW22 was grown in alkaline meat extract broth containing various concentrations of iron. No reduction of hemolytic activity was observed below 100 μM iron. However, at higher concentrations, an inverse relationship between medium iron and hemolytic activity was found (Fig. 4). Addition of 1 mM iron to previously formed hemolysin did not reduce the hemolytic activity. Therefore, it is likely that the decrease in hemo-

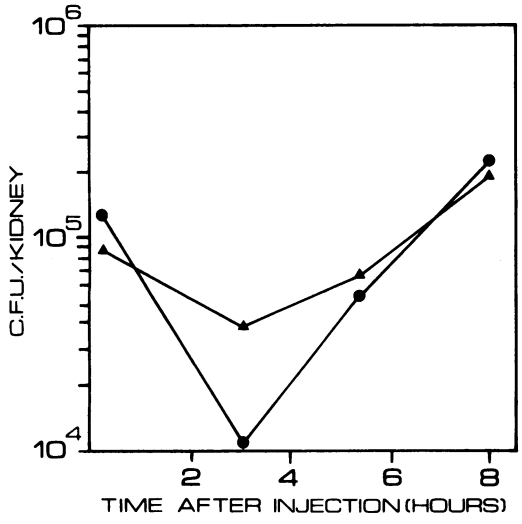

FIG. 3. Total kidney viable count pattern of strain CW101 after intraperitoneal injection of 1.4 μM ferrous sulfate (▲) or 1.4 μM sheep hemoglobin (●). Two groups of mice were injected with either hemoglobin or $FeSO_4$. Within the observation period (48 h), they did not show any sign of illness. Each symbol represents the mean viable count in both kidneys of two mice.

lytic activity was caused by reduced production of hemolysin.

DISCUSSION

The aim of the present study was to determine the function of hemolysin in the nephropathogenicity for mice of *E. coli* CW22. Previously, we have shown that the ability to produce hemolysin is essential for the nephropathogenicity of this strain (20a, 21). The results presented here suggest that hemolysin enhanced the nephropathogenicity by lysis of erythrocytes. The liberated hemoglobin subsequently acted as a source of iron, which is necessary for bacterial growth.

The enhancement of virulence by hemolysin was shown by our finding that the nonhemolytic, avirulent strain CW101 was rendered nephropathogenic by the simultaneous injection of either its hemolytic parent or partially purified hemolysin.

Using a mouse model that measures general virulence, Linggood and Ingram (17) obtained similar results. Their results, however, may be explained in several ways. First of all, the enhanced virulence of avirulent *E. coli* strains in the presence of hemolysin could be caused by the detrimental effect of hemolysin on the bacterial killing by polymorphonuclear leukocytes. At low doses of hemolysin, the majority of leukocytes remain viable. However, attachment, ingestion, and intracellular killing are highly impaired under these conditions (6, 7). By circumvention of the host defense in this manner, hemolysin would confer a general virulence to nonpathogenic *E. coli* strains.

Secondly, hemoglobin, the major product of the hemolytic reaction, could interfere with the phagocytic destruction of *E. coli*. The chemotactic response of leukocytes has been shown to be inhibited by hemoglobin, both in vitro and in vivo (14, 15). However, such an interference could not be confirmed by Eaton et al. (9).

Thirdly, the iron moiety of hemoglobin may influence the virulence of *E. coli* strains in different ways. The enhancement of bacterial virulence under hyperferremic conditions is well recognized (for reviews, see references 3, 22, and 23). Two mechanisms could explain this phenomenon. Increased levels of iron stimulate bacterial growth on the one hand and impair host defense on the other.

An *E. coli* strain that was killed in fresh serum showed rapid growth in the same serum after the addition of iron (10). The impairment of leukocyte functions by the addition of iron is believed to act in several ways. Increased levels of iron saturate intracellular lactoferrin, resulting in the survival of ingested bacteria, without interfering with phagocytosis (4). In in vitro experiments, Gladstone and Walton showed that the bactericidal effect of basic bactericidal proteins from lysosomes could be reverted by the addition of iron (12). Finally, it has been suggested that the destruction of hydrogen peroxide by iron also prevents the killing of bacteria (16).

From these results, the central role of iron in the host-parasite interactions is evident. Although these mechanisms are not mutually ex-

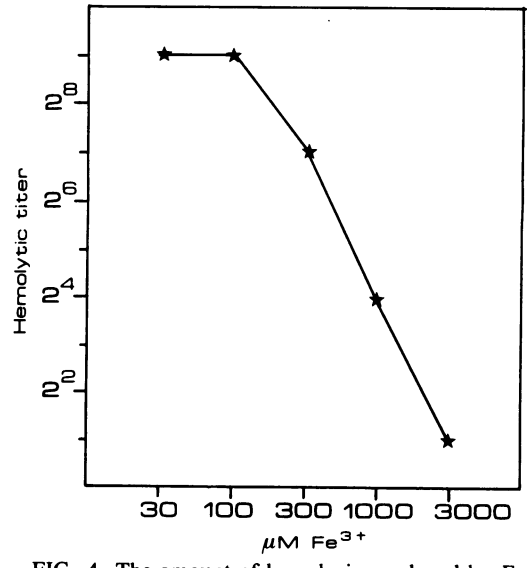

FIG. 4. The amount of hemolysin produced by *E. coli* CW22 as a function of medium iron.

clusive, a virulence model that measures only general virulence cannot discriminate between advantage to the parasite and disadvantage to the host, conferred by increased levels of iron or hemoglobin.

The results of the present study, on the other hand, showed that iron enhanced the virulence for mouse kidneys. These results suggest that the impairment of leukocyte functions is only secondary to the stimulation of bacterial growth. In addition, others have shown that iron can provoke experimental pyelonephritis in rats, whereas the clearance of *E. coli* from other organs is not affected (11). Furthermore, experimental pyelonephritis is exacerbated by administration of iron (13).

The enhanced nephropathogenicity by simultaneous injection of hemoglobin can also be explained by growth stimulation. In this paper, we demonstrated that hemoglobin can support bacterial growth under iron-restricted conditions in vitro, whereas Bornside et al. (2) showed that *E. coli* multiplies rapidly in vivo in the presence of hemoglobin. Furthermore, growth stimulation by hemoglobin is abolished both in vitro and in vivo by simultaneous administration of haptoglobin (9). The level of this naturally occurring hemoglobin-binding protein is increased 10-fold as a response to inflammation (1). This mechanism, together with decreased intestinal absorption of iron and increased storage in the liver immediately after initiation of infection (22, 23), adds to the nutritional immunity of the host by lowering the amount of available iron.

In the case of hemolysin, similar arguments can be used to explain the enhanced nephropathogenicity. In addition, kidney infection may be exacerbated by renal lysosome disruption by *E. coli* hemolysin (8). If this would occur in vivo, lysosomal enzymes could damage renal parenchym cells, providing a nidus for bacterial multiplication. This latter finding may well be of importance in ascending infections, which are believed to be the main route of infection of the urinary tract in humans. However, our finding that exogenous iron inhibits hemolysin production in vitro supports the idea that virulence enhancement of hemolysin in hematogenous pyelonephritis functions mainly by increasing the level of available iron.

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