Influence of Iron on Corynebacterium renale-Induced Pyelonephritis in a Rat Experimental Model

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Growth of Corynebacterium renale in vitro on low-iron medium (1.34 μM) was only slightly less than that on high-iron media (7.16 and 9.85 μM). However, studies on C. renale-induced pyelonephritis using the rat as an experimental model revealed that C. renale cultivated in high-iron media was capable of producing pyelonephritis, but when grown on low-iron medium, these bacteria were noninfective. This apparent avirulence of the bacteria cultivated on low levels of iron could be reversed by injecting the rats intramuscularly with ferric ammonium citrate.

Corynebacterium renale is isolated primarily from cattle, where it produces a naturally occurring cystitis and ascending pyelonephritis (1). In an attempt to facilitate the study of the pathogenesis of C. renale-induced pyelonephritis, a rat experimental model was developed (9), and it was shown that the gross appearance and histological condition of the renal tissue of infected rats bore a close resemblance to that of the renal tissue of cows exhibiting signs of pyelonephritis. In addition, experimental infection in rats could be prevented (10) by the daily oral administration of the urease inhibitor acetohydroxamic acid, and evidence was presented to indicate that the powerful urease possessed by C. renale (12, 15) could play a role in the pathogenesis of this disease.

However, since acetohydroxamic acid is a simple monohydroxamic acid (3) and, like all hydroxamates, has the ability to chelate iron (14), it was also anticipated that acetohydroxamic acid could be exerting its in vivo effect to some extent by depleting the iron content of the host. Nutritional immunity, the ability of a host to deprive an invading microorganism of essential nutrients, is a recognized factor in the establishment of infections (22). Bacteria must be able to obtain sufficient levels of iron for in vivo growth, possibly by the production of their own iron chelators (21).

This study was initiated to determine if the iron content of the C. renale cultivation medium had any effect on the in vitro growth of these bacteria or on their ability to produce pyelonephritis in rats. It has been reported previously (6) that no growth or poor growth of each of several different strains of C. renale was observed in minimal medium without added iron.

Also, injection of iron compounds into a number of host animals susceptible to various bacterial infections has been shown to stimulate bacterial growth in vivo, accompanied by a drastic reduction in the 50% lethal dose for rats (5, 16). Consequently, this study was initiated to determine if parenteral injections of iron into rats, in the form of ferric ammonium citrate, would allow C. renale grown in a low-iron medium to establish an acute pyelonephritis.

MATERIALS AND METHODS

Test organism. C. renale strain 1321, which was originally isolated at the Teaching Hospital, College of Veterinary Medicine, University of Georgia, Athens, from a cow exhibiting signs of pyelonephritis, was used for this study.

Media and growth conditions. The bacteria were cultivated in the following three media: (i) brain heart infusion broth (BHI), (ii) a chemically defined synthetic medium developed by Hirai and Yanagawa (7) and Hirai et al. (6) to which was added ferric citrate instead of ferrous sulfate and which was designated as complete medium, and (iii) this same synthetic medium without ferric citrate.

Growth was measured in optically matched HyceI tubes containing 10 ml of BHI, after inoculation with 0.1 ml of a 48-h culture of C. renale, and incubation at 37°C with shaking on a New Brunswick gyratory shaker (model G-2) at 150 rpm. Turbidity of the cultures was measured as the change of light transmission (in percentage) as compared with an un inoculated control tube with a Coleman model 14 spectrophotometer at a wavelength of 630 nm. A standard curve was constructed by correlating viable cell counts, determined by plate counts on blood agar, with the percentage of change of light transmission. Each trial consisted of a series of nine tubes, and trials were performed in triplicate before values were averaged to obtain the curve. The same procedure was followed to
construct curves for growth on complete medium with or without ferric citrate.

Cultures maintained on complete medium with or without ferric citrate and starting out with $3.24 \times 10^6$, $3.24 \times 10^7$, $3.24 \times 10^8$, $3.24 \times 10^9$, or $3.24 \times 10^{10}$ colony-forming units (CFU)/ml were used in an effort to determine the effect of inoculum size on the growth of C. renale in medium with or without added iron.

**Iron determinations.** All three media used for the cultivation of C. renale were analyzed for iron content by atomic absorption spectroscopy, using a Perkin-Elmer model 305A atomic absorption spectrophotometer. Absorbance was measured at 248.3 nm, using acetylene and air flame. Cells cultivated in complete medium with or without ferric citrate were washed three times with glass-distilled water after centrifugation at 11,000 rpm in a Sorvall RC2-B refrigerated centrifuge equipped with a GS-3 rotor. The cell samples were then incubated at 60°C overnight, and the ashed material was dissolved in 10% hydrochloric acid. The iron content of each sample was measured as described above and expressed as micrograms of iron per gram of dry weight. The dry weight determinations were conducted in crucibles that were soaked overnight in nitric acid and rinsed thoroughly in glass-distilled water.

**Maintenance and infection of rats.** Charles River outbred male albino rats were housed in metabolic cages which had food and water facilities separated from the cage interior by a short tunnel apparatus. Water and rat chow pellets, which had been pulverized to powder, were available ad libitum.

C. renale cells, at a concentration of approximately $10^6$ cells per ml, were centrifuged at 1,085 $\times g$ for 10 min in a model RC2-B Sorvall refrigerated centrifuge and then resuspended in 10 ml of 0.85% sodium chloride. Washing was repeated twice, and the cells were resuspended in a final volume of 1 ml of saline. Zinc disks, 6 mm in diameter, were dipped into the bacterial suspension, and each disk carried approximately $10^6$ CFU of C. renale. These were used to infect rats weighing 150 to 250 g by the method of Vermeulen and Goetz (18).

Anesthesia was achieved by the intraperitoneal injection of sodium pentobarbital. The urinary bladder was exposed by a suprapubic incision of the abdominal wall. The zinc disks coated with C. renale were inserted directly into the bladder after incision of the bladder wall. Sterile disks were implanted into the bladders of control rats.

**Monitoring of disease process.** Necropsies were performed either after euthanasia or upon the death of the animal. Both kidneys were removed under aseptic conditions and bisected longitudinally. The urinary bladder and one-half of each kidney were fixed in 10.0% buffered Formalin, sectioned at 5 to 7 μm, stained with hematoxylin and eosin, and examined microscopically. Selected renal tissue was stained by the Perls method for iron as described previously (13).

The other kidney halves were placed in sterile VirTis homogenizing jars, weighed, and suspended in 20 ml of physiological saline. The sections were then homogenized with a VirTis homogenizer model 23 (6-105AF) over an ice bath. A 1.0-ml sample was then aseptically removed from the jar, serially diluted, and plated onto blood agar. These plates were incubated for 24 and 48 h and examined for the presence of C. renale.

**Administration of ferric ammonium citrate.** All infections followed the regimen of one injection per day; beginning 24 h post surgery, as described by Fletcher and Goldstein (5), until necropsy after 7 days. Cultures of C. renale used to infect rats at surgery were grown only in complete medium without ferric citrate. Test animals received ferric ammonium citrate injections in the biceps femoris at a concentration of 10 mg/kg of body weight. Negative controls were noninfected rats receiving injections of ammonium citrate or ferric ammonium citrate. Positive controls were infected animals which received injections of ammonium citrate, saline, or no injections. Both control groups received injections according to the same regimen as the test animals.

**Statistical analysis.** The number of C. renale per gram of kidney was converted to log10, and the mean was determined for each test group. The data were analyzed using an analysis of variance (one-way with equal or unequal replication) with the final intention of comparing the means by the method of least significant difference.

**RESULTS**

**Effect of varying iron concentration on C. renale growth in vitro.** C. renale was cultivated on three types of media to determine the effect that varying the iron content of the media had on the growth of these bacteria. The iron concentrations of BHI, complete medium with ferric citrate (high-iron medium), and complete medium without ferric citrate (low-iron medium) were 9.85, 7.16 and 1.34 μM, respectively. Studies in vitro showed that growth in BHI was only slightly higher than that in complete medium with added iron (Fig. 1). This observation correlates well with the iron concentrations of the respective media. However, the complete medium without added iron, which had a dras-

![Fig. 1. Comparison of growth by C. renale in BHI, complete medium with ferric citrate, and complete medium without ferric citrate. All values are averages of three experiments.](image-url)
tically reduced iron content in comparison to the other media, yielded bacterial growth which was not appreciable lower. The initial cell number of these cultures was $3.24 \times 10^8$ CFU/ml. When the inoculum size was reduced 10-fold, growth on low-iron medium was about 15% less than that on high-iron medium. This quantitative difference in growth on synthetic medium containing two different levels of iron remained the same when the inoculum size was even smaller, namely, $3.24 \times 10^6$, $3.24 \times 10^4$, $3.24 \times 10^2$, or $3.24 \times 10^0$ CFU/ml.

Measurements of the iron content of cells cultivated in high- and low-iron media revealed that the former contained 1,013 µg and the latter 296 µg of iron per g of dry weight.

**Infection of rats with C. renale cultivated on high-iron and low-iron media.** Studies in vivo were initiated to determine if there was a more pronounced requirement for iron by C. renale to produce pyelonephritis in rats than had been noted in the in vitro growth investigations. It can be observed from Table 1 that the iron content of the media had a definite effect on the number of C. renale recovered from the renal tissue. Kidneys of rats infected with cells cultivated on BHI or complete medium with ferric citrate yielded plate counts approximately 1.5 logs higher than kidneys of rats infected with cells cultivated on low-iron medium. The difference between the BHI and the low-iron groups, as well as that between the complete medium with ferric citrate and the low-iron groups, was significant ($P < 0.01$). On the other hand, the difference between the BHI and the complete medium with ferric citrate groups was not significant ($P > 0.05$).

Microscopic examination of rat kidney showed the effect that the iron concentration of the media had on the ability of C. renale to produce pyelonephritis. Noninfected control rats, which had only sterile disks implanted in their urinary bladders, had no remarkable renal lesions (Fig. 2). In comparison to the controls, kidneys from rats infected with cells grown in BHI had lesions characterized as an acute necrotizing supplicative nephritis (Fig. 3). The transitional epithelial lining of the pelvis was eroded, and marked necrosis extended from the pelvis into the medulla. Tubules in the medulla had undergone granular degeneration, and leukocytes often infiltrated the medulla. Tissue from kidney infected with C. renale grown in high-iron medium had lesions of pyelonephritis similar to kidneys infected with BHI-grown cells. However, rat kidneys infected with cells grown in low-iron me-

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**Table 1.** Number of C. renale in kidneys of infected rats as a function of iron content of cultivation media

<table>
<thead>
<tr>
<th>Cultivation medium</th>
<th>No. of C. renale* in kidney (log$_{10}$ CFU/g of kidney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninfected*</td>
<td>0.0</td>
</tr>
<tr>
<td>BHI</td>
<td>6.95 ± 0.93</td>
</tr>
<tr>
<td>Complete, with iron</td>
<td>7.22 ± 0.22</td>
</tr>
<tr>
<td>Complete, without iron</td>
<td>5.73 ± 0.21</td>
</tr>
</tbody>
</table>

* Ten animals used for each test group.

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**Fig. 2.** Photomicrograph of renale tubules in medulla. Hematoxylin and eosin; ×250.

**Fig. 3.** Photomicrograph of necrosis and granular degeneration of renal tubules in the medulla of a rat infected with C. renale grown in BHI. Hematoxylin and eosin; ×250.
dium had no lesions of pyelonephritis and were structurally similar to those of noninfected rats.

**Effect of administration of ferric ammonium citrate to rats on *C. renale*-produced pyelonephritis.** Since *C. renale* appeared to be unable to produce pyelonephritis when grown in medium where the iron content was below an undetermined critical level, an attempt was made to establish if iron, supplied by parenteral injection of ferric ammonium citrate, would provide sufficient iron for cells cultivated on low-iron medium to establish an acute infection. The results of experiments designed to detect the number of *C. renale* cells in kidneys of infected rats as a function of iron administration are shown in Table 2.

Whereas no *C. renale* could be detected in the renal tissue of noninfected rats, the rats infected with *C. renale* and injected with saline, ammonium citrate, or not injected at all yielded plate counts of approximately 

\[ \log_{10} \text{CFU/g of kidney} \]

The test rats, which received ferric ammonium citrate injections, yielded plate counts 2 to 2.5 logs higher than each of the controls. The difference between the ferric ammonium citrate group and each of the control groups was significant (\( P < 0.01 \)). The difference between the saline and ammonium citrate groups was not significant (\( P > 0.05 \)).

Microscopic examination of tissue of the negative controls, which were not infected with *C. renale*, showed no renal damage due to the injected ammonium citrate or ferric ammonium citrate. Positive control rats receiving saline injections also demonstrated normal renal tissue, as seen in noninfected control rats (Fig. 2). In comparison, test rats receiving ferric ammonium citrate exhibited extremely severe pyelonephritic lesions. The renal lesions in these rats were remarkably similar to those of rats infected with cells grown in BHI (Fig. 3) or high-iron complete medium.

Sections of renal tissue were also stained by the Perls method for iron (13). Negative controls, receiving ferric ammonium citrate, had heavy staining for iron in the renal tubules (Fig. 4). An iron-staining procedure was done also on kidneys from rats which had zinc disks, coated with *C. renale* cultivated on low-iron medium, implanted in their urinary bladders. These rats developed pyelonephritis after intramuscular injection of ferric ammonium citrate, but no iron was detected in renal tissue stained with Perls stain.

**DISCUSSION**

Varying the iron concentration in growth media has been demonstrated by a number of investigators to have profound effects on microbial cell metabolism. Observations on iron-deficient batch cultures have revealed that the quantity of cellular material was reduced (11). Studies of the enzymes of the iron-deficient cells showed that the concentration of the enzymes possessing iron porphyrin prosthetic groups was decreased. These are only isolated examples of a whole host of changes that have been reported to occur in bacterial as well as fungal cells growing in media deprived of an adequate amount of iron. Consequently, it was anticipated that varying the iron concentration of the *C. renale* growth media from an iron-sufficient level (9.85 or 7.16 μM) to

![Fig. 4. Photomicrograph of the renal tubules of the cortex from a noninfected control rat receiving injections of ferric ammonium citrate. Observe the granules (arrows) indicating positive staining for iron in the cytoplasm of epithelial cells of renal tubules. Perls stain; ×900.](image-url)
a much lower level (1.35 μM) could conceivably lead to drastic changes in the growth rate and final cell yield of C. renale. However, when cultivated in a low-iron defined medium, this bacterium was found to grow at only a slightly reduced level (10 to 15%) when compared with its growth on BHI or high-iron defined medium. Plate counts showed less than 1-log difference in growth on the three levels of iron used. Of course, there is the distinct possibility that the concentration of iron in the cultivation medium used for the studies described herein may not have been rate limiting, since it has been determined that the quantity of iron needed for good growth of commonly studied bacteria may vary between 0.358 and 3.58 μM (20).

Nevertheless, it was suspected that there might be a more pronounced requirement for iron by C. renale in vivo and that iron may in fact be a virulence factor for the production of pyelonephritis by C. renale. Growth of C. renale in vivo was found to be definitely different when the organism was cultivated in media containing different concentrations of iron. When grown in low-iron defined medium, C. renale proved to be avirulent for rats, but when cultivated in BHI or high-iron defined medium, the organism was extremely virulent. Since iron concentration was the only factor which varied in each group, it seemed reasonable to assume that iron was serving a role as a “virulence factor.”

Additional credence for this hypothesis stemmed from experiments involving the development of pyelonephritis in rats that had been given ferric ammonium citrate parenterally 24 h after implantation in the urinary bladders of zinc disks coated with C. renale cultivated in low-iron complete medium. Previous investigations by Fletcher and Goldstein (5) had shown that injection of low-molecular-weight compounds such as iron sorbitol citrate or ferric ammonium citrate enhanced kidney infections in mice by Escherichia coli, Mycobacterium fortuitum, and avirulent Staphylococcus albus. Moreover, in the studies with C. renale, it was noted that when the renal tissue of experimental rats was treated with Perls stain, ample stain could be detected within the renal tubules of noninfected rats injected with ferric ammonium citrate, whereas little if any stain was found in the tubules of C. renale-infected rats that received ferric ammonium citrate. This may indicate that the C. renale cells utilized the iron supplied to the host by injection to produce pyelonephritis.

C. renale cells grown in high-iron medium and capable of producing pyelonephritis contain approximately 3.5 times as much iron as those cells cultivated in low-iron medium and which are not able to initiate pyelonephritis. The iron concentration of bacterial cells as a function of its concentration in the medium has been demonstrated for Aerobacter indologenes (19), Corynebacterium diphtheriae (4), and Shigella dysenteriae (17). The capacity of some bacterial cells to store more iron than they require to fulfill immediate needs seems to give them an advantage when the iron in their environment is decreased drastically. In this event, there may be a redistribution of the iron in the cell to serve more critical cell functions. In the case of C. renale, it is conceivable that the large amount of iron stored by the cells grown in high-iron medium as compared with that stored by cells cultivated in low-iron medium may be one factor in explaining why the former are capable and the latter incapable of producing pyelonephritis.

The experiments of this study taken as a whole seem to indicate that to produce pyelonephritis in rats, C. renale cells must be supplied with iron above a certain undetermined critical level. The mechanism by which the iron acts is not known. It may function as a bacterial growth-promoting factor, with the required iron concentration being higher for in vivo than for in vitro growth, especially since the bacteria must compete with the host’s iron-binding proteins such as lactoferrin and transferrin for in vivo iron. Iron has also been shown to increase adherence to spermatozoa by gonococci (8), and a similar adherence enhancement of C. renale to the urinary tract mucosa and/or the renal tissue may occur in renal infections. It has already been reported that iron inhibits intracellular killing after phagocytosis (22), and this may explain the reduction of the 50% lethal dose for rats that was obtained with parenteral injection of iron compounds in the infections discussed.

Iron may also depress host resistance in the kidneys, since in vivo inhibition of immune mechanisms has been reported after iron injections (2). However, large doses of low-molecular-weight compounds, such as ferric ammonium citrate, do not affect renal function or structure. Also, blockage of the reticuloendothelial system is not an explanation. Iron dextran, which is taken up by the reticuloendothelial system, does not alter renal resistance to infection. Ferric ammonium citrate does not block the reticuloendothelial system, but it does, nonetheless, alter renal resistance to infection (5). However, inhibition of the immune mechanisms would not explain the fact that C. renale cells grown in BHI or high-iron complete medium were infective whereas those cultivated in low-iron complete medium were not infective.

ACKNOWLEDGMENTS

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absorption spectroscopy and his contribution to this study is deeply appreciated. Statistical analysis for the data pertaining to the C. renale CFU per gram of kidney in the various test groups was done by John Brown, and his assistance is gratefully acknowledged.

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LITERATURE CITED