THE ROLE OF IRON IN NONSPECIFIC RESISTANCE TO INFECTION INDUCED BY ENDOTOXIN

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Studies using an experimental model of the mouse and Candida albicans as the challenge organism attempted to define better the mechanism of nonspecific resistance to infection induced by bacterial endotoxins. In vitro studies showed a positive correlation between the growth of C. albicans in serum from mice obtained at daily intervals for 10 days after endotoxin or saline injection and the percentage iron saturation of the serum. In vivo studies in which one of four different concentrations of iron as ferric ammonium citrate was injected into control and endotoxin-treated mice at the time of challenge with C. albicans showed that the rate of mortality of the mice was directly related to the concentration of iron injected. The endotoxin-induced nonspecific resistance was negated and reversed by iron administration. These studies demonstrate that changes in iron metabolism induced by bacterial endotoxin are a key factor in the mechanism of resistance to C. albicans infection in mice.

In 1955, the observation was made that mice had a greatly reduced susceptibility to several different strains of Escherichia coli if the mice had been injected with E. coli cell walls 24 hr before challenge with the bacteria (1). Since that time, it has been shown that a variety of bacterial endotoxins will induce resistance to infection with some bacteria (Escherichia coli, Klebsiella, Pneumococci, Salmonellae and Staphylococci); fungi (Candida and Cryptococcus); parasites (Plasmodia and Trypanosoma); and viruses (Ectromelia, encephalitis, and Newcastle disease) as the challenge organisms (1–12). A recent review of the effects of endotoxins on susceptibility to infections indicated the increased resistance may be related to enhanced macrophage and reticuloendothelial system activity, granulocytosis, and vascular changes (13). However, the mechanism of this nonspecific resistance to infection secondary to endotoxin administration remains unclear.

Previous studies in this laboratory demonstrated that sera obtained from mice 24 hr after endotoxin injection did not support the growth of Candida albicans as well as normal sera (6). Since it is known that iron is an essential growth factor for C. albicans (14, 15) and that endotoxin causes a significant fall in serum iron (16), the importance of iron as a factor in nonspecific resistance to infection with C. albicans induced by endotoxin was evaluated in vitro and in vivo.

MATERIALS AND METHODS

Experimental animals. Male Swiss white mice from the National Institutes of Health colony weighing between 18 and 24 g were used in all studies.

Organism. Strain B311 of C. albicans, originally isolated from the blood of a patient with fatal disseminated candidiasis, was used. Inocula were prepared from organisms grown in Sabouraud’s broth for 18 hr at 37°C with constant agitation. The yeast cells were harvested, washed three times with pyrogen-free, 0.85% phosphate-buffered saline (PBS). After direct counting in a hemocytometer, the inoculum was adjusted to the desired concentration of organisms with PBS. In addition, serial 10-fold dilutions of the inoculum were plated on Sabouraud’s agar to determine viable organisms. A very close agreement between the

1 Abbreviations used in this paper: PBS, phosphate-buffered saline; UIBC, unbound iron-binding capacity; TIBC, total iron-binding capacity.
hemocytometer count and colony count was found.

**Endotoxin.** An endotoxin purified from *Escherichia coli* 0111:B4 (lot 541700, Difco Laboratories, Detroit, Mich.) with a Westphal extraction was prepared in a concentration of 200 μg/ml with PBS and stored at -20°C.

**Iron determination.** The serum iron and unbound iron-binding capacity were determined colorimetrically according to the method of Schade et al. with bathophenanthroline as the color reagent (17).

**Serum collection.** Mice were aseptically exsanguinated by transection of the axillary artery and vein. The blood was pooled, allowed to clot, and the serum was separated by centrifugation. Sterility was confirmed by culturing a sample of serum in thioglycolate broth. Only sterile sera were used for the *in vitro* studies. All sera were stored at -20°C before use.

**In vitro experimental design.** Six hundred mice were each injected i.p. with 200 μg of endotoxin and another group of 600 mice was injected i.p. with 1 ml of saline. Sixty mice were bled each day for the next 10 days. The blood of 15 mice was pooled for one sample or four samples were obtained each day from the endotoxin-treated and saline groups. A group of 60 mice that did not receive endotoxin or saline was bled to serve as time 0 samples. All mice were bled in the morning at approximately the same time of day. The serum iron and unbound iron-binding capacity (UIBC) were determined on each serum sample. The *in vitro* growth of *C. albicans* in the mouse sera was evaluated by the following test solution:

**Composition**

1.0 ml mouse sera

0.1 ml containing Tris, dextrose and chloramphenicol at pH 8.1

0.1 ml containing *C. albicans* in saline

**Final concentration**

*C. albicans*, 10⁶ organisms/ml

Dextrose, 100 mg/100 ml

Tris buffer, 0.1 M

pH, 8.1

Dextrose was added to eliminate its deficiency as a possible growth-limiting factor. Chloramphenicol prevented bacterial overgrowth from an inadvertent bacterial contamination during the preparation of the test solutions. The pH was controlled with Tris (2-amino-(2-hydroxymethyl)-1,3-propane-diol) since it has been shown that the growth of *C. albicans* in serum is inversely related to pH (18). All the test solutions were incubated simultaneously with the same *Candida* inoculum at 37°C in sterile cotton-stoppered test tubes with constant circular agitation at a rate of 100 rpm by using a gyratory incubator-shaker (model G25, New Brunswick Scientific Co., New Brunswick, N. J.). After 24 hr of incubation, duplicate 0.1-ml samples were removed from each test tube and the colony count was determined by serial 10-fold dilutions and growth on Sabouraud’s agar. All pour plates were counted with a Darkfield Quebec colony counter (American Optical Company, Buffalo, N. Y.). A fixed smear from each sample stained with methylene blue showed less than 0.1% of the organisms in the mycelial phase. Clumping of *Candida* as described by Louria et al. (19) and Children et al. (20) was not observed with phase microscopy in the present study. The explanation for the latter finding may be due to different incubation conditions, small inocula, and the use of murine serum in the experiments reported below. Therefore, each colony counted was assumed to represent one *C. albicans* organism. The pH of each test solution was measured with a pH meter (Expandomatic SS-2, Beckman Instruments, Inc., Fullerton, Calif.) after incubation and ranged from 8.04 to 8.12.

Iron in the form of ferric ammonium citrate was added to an aliquot of the 1-day post-endotoxin serum samples to achieve a percentage iron saturation of 75%. These serum samples were also evaluated in the above described test solution for growth of *C. albicans*.

**In vivo experimental design.** Twelve groups of 25 mice were injected i.p. with either 200 μg of endotoxin or saline. After 24 hr, all mice were challenged with 10⁴ *C. albicans* i.v. In addition, the mice were given an i.p. injection of 1 ml of either one of four different concentrations of ferric ammonium citrate (containing 50 μg, 100 μg, 150 μg or 200 μg of iron/ml) or ammonium citrate (containing 806 μg/ml) or saline. After inoculation, mice were observed at 12-hr intervals and the number of surviving mice was tabulated. Deaths within the first 6 hr were considered to be due to technical causes and these animals were excluded from the study. Also, 800 mice were injected i.p. with 1 ml of one
of the four concentrations of ferric ammonium citrate or ammonium citrate. Groups of 32 mice (the blood of 8 mice was pooled for 1 serum sample or 4 samples per group) were bled at 1, 2, 4, 6, and 24 hr after injection and the serum iron and UIBC were determined. Three control groups of 25 mice were injected i.p. with either 200 μg of endotoxin, 200 μg of iron as ferric ammonium citrate or 200 μg of endotoxin followed by 200 μg of iron 24 hr later. These three groups were observed for deaths for 10 days.

Statistics. The number of *C. albicans* per 0.1 ml of test solution (N) was estimated by the following formula:

\[ N = \frac{C_1 + C_2 + \ldots + C_m}{D_1 + D_2 + \ldots + D_m} \]

where \( C_i \) (\( i = 1, 2, \ldots, m \)) is the colony count of the \( i \)th dilution and \( D_i \) is the \( i \)th dilution factor, namely \( D_i = 10^{-i} \). A minimum of eight observations was obtained for each average colony count (N). The mean and standard error of the mean (S.E.M.) were calculated for the four samples at each time interval. Groups were compared by applying Student's t-test. Linear regression analysis and the Spearman rank correlation coefficient were used to evaluate the relationship between iron concentration and colony count (21). Statistical analysis of the mortality data was performed by the Wilcoxon test (22).

**RESULTS**

The growth of *C. albicans* in mouse sera paralleled the changes in serum iron and percentage iron saturation over a 10-day period after a single injection of endotoxin or saline into donor mice (Figs. 1, 2). The relationship was especially close during the first 5 days after endotoxin administration. A cyclical pattern of

![Graph](http://example.com/graph.png)

*Figure 1.* Temporal relationship of the percentage iron saturation and growth of *C. albicans* in mouse sera obtained at daily intervals for 10 days after the i.p. injection of 200 μg of endotoxin (circles) or saline (triangles). The colony count of *C. albicans* in 0.1 ml of test solution (interrupted line) is on the right ordinate and the percentage iron saturation (solid line) on the left ordinate. Time 0 represents control mice (squares) that received no injection. Each point represents the mean of four observations.
the serum iron and percentage iron saturation occurred after a single injection of endotoxin with a statistically significant fall (p < 0.05) at 1 day, 4 days, and 7 days compared with serum from saline-injected animals (Table I). Spearman rank correlation coefficients were calculated for each value of the colony count compared to the percentage iron saturation and serum iron, respectively, (Fig. 3 and Table II). These coefficients show a significant correlation between the colony count and the percentage iron saturation and, to a lesser degree, between the colony count and serum iron concentration. The addition of iron to 1-day mouse sera to achieve 75% iron saturation restored the growth properties of these sera (5075 ± 762 colonies/0.1 ml) to that of normal mouse sera (time 0) and significantly abolished the growth inhibition of these 1-day mouse sera (p < 0.05).

The i.p. injection of iron at the time of challenge of 1-day post-endotoxin-treated and control mice with *C. albicans* clearly increased the rate of mortality (Fig. 4). This phenomenon was dose dependent; thus, increasing amounts of iron effected a progressive increase in the rate of mortality of control and endotoxin-treated mice (Figs. 4, 5). At 200 μg of iron, there was virtually a complete reversal of the endotoxin-induced resistance to *Candida* infection (Fig. 5). The mortality rates of the saline and ammonium citrate groups were essentially identical. One animal out of 25 died in each of the control groups injected only with endotoxin and endotoxin plus iron 24 hr later. No deaths were observed in the control group of animals receiving only iron.

The serum iron and percentage iron saturation at five time intervals after the i.p. injection of ferric ammonium citrate or ammonium citrate are shown in Table III. Free serum iron (percentage iron saturation greater than 100%) occurred only in the animals receiving 200 μg of
TABLE I
Changes in the growth of Candida albicans and iron concentration in mouse sera after endotoxin or saline administration

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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</thead>
<tbody>
<tr>
<td><strong>Endotoxin</strong></td>
<td>Colony count (in 0.1 ml)</td>
<td>2450</td>
<td>3230</td>
<td>5032</td>
<td>3074</td>
<td>4879</td>
<td>3203</td>
<td>2602</td>
<td>5889</td>
<td>4456</td>
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<td>474</td>
<td>263</td>
<td>974</td>
<td>418</td>
<td>801</td>
<td>363</td>
<td>514</td>
<td>553</td>
<td>605</td>
</tr>
<tr>
<td><strong>Saline</strong></td>
<td>Colony count (in 0.1 ml)</td>
<td>5653</td>
<td>5175</td>
<td>4867</td>
<td>5195</td>
<td>5012</td>
<td>5388</td>
<td>5577</td>
<td>5500</td>
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<td>514</td>
<td>618</td>
<td>474</td>
<td>519</td>
<td>493</td>
<td>701</td>
<td>582</td>
<td>955</td>
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<td>0.1</td>
<td>0.9</td>
<td>0.05</td>
<td>0.7</td>
<td>0.025</td>
<td>0.01</td>
<td>0.7</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Endotoxin</strong></td>
<td>Serum iron (µg/100 ml)</td>
<td>100.0</td>
<td>249.5</td>
<td>242.8</td>
<td>173.6</td>
<td>268.3</td>
<td>293.2</td>
<td>215.6</td>
<td>237.9</td>
<td>275.7</td>
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<td>24.1</td>
<td>15.8</td>
<td>6.3</td>
<td>11.6</td>
<td>3.7</td>
<td>11.6</td>
<td>6.1</td>
<td>23.9</td>
</tr>
<tr>
<td><strong>Saline</strong></td>
<td>Serum iron (µg/100 ml)</td>
<td>275.5</td>
<td>318.6</td>
<td>289.1</td>
<td>265.4</td>
<td>286.9</td>
<td>303.8</td>
<td>310.4</td>
<td>308.4</td>
<td>314.4</td>
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<td></td>
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<td>18.1</td>
<td>22.9</td>
<td>18.6</td>
<td>19.9</td>
<td>13.3</td>
<td>10.8</td>
<td>14.8</td>
<td>8.3</td>
<td>11.4</td>
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<tr>
<td>P value (&lt;)</td>
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<td>0.3</td>
<td>0.5</td>
<td>0.001</td>
<td>0.1</td>
<td>0.4</td>
<td>0.001</td>
<td>0.7</td>
<td>0.005</td>
<td>0.7</td>
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<tr>
<td><strong>Endotoxin</strong></td>
<td>TIBC (µg/100 ml)</td>
<td>307.9</td>
<td>419.2</td>
<td>319.8</td>
<td>410.6</td>
<td>468.3</td>
<td>490.1</td>
<td>439.4</td>
<td>371.7</td>
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<td>13.1</td>
<td>16.9</td>
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<td>11.7</td>
<td>12.2</td>
<td>6.0</td>
<td>25.7</td>
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<tr>
<td><strong>Saline</strong></td>
<td>TIBC (µg/100 ml)</td>
<td>359.2</td>
<td>381.4</td>
<td>356.4</td>
<td>311.3</td>
<td>359.9</td>
<td>371.6</td>
<td>363.1</td>
<td>359.3</td>
<td>366.4</td>
</tr>
<tr>
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<td>15.6</td>
<td>23.4</td>
<td>22.7</td>
<td>23.7</td>
<td>15.4</td>
<td>17.6</td>
<td>3.5</td>
<td>8.9</td>
</tr>
<tr>
<td>P value (&lt;)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.05</td>
<td>0.2</td>
<td>0.005</td>
<td>0.001</td>
<td>0.001</td>
<td>0.7</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Endotoxin</strong></td>
<td>% saturation (%)</td>
<td>34.2</td>
<td>59.1</td>
<td>62.0</td>
<td>42.6</td>
<td>57.2</td>
<td>59.8</td>
<td>49.0</td>
<td>63.9</td>
<td>73.4</td>
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<tr>
<td></td>
<td>S.E.M.</td>
<td>7.8</td>
<td>4.7</td>
<td>3.8</td>
<td>3.0</td>
<td>1.9</td>
<td>2.0</td>
<td>2.2</td>
<td>2.6</td>
<td>3.9</td>
</tr>
<tr>
<td><strong>Saline</strong></td>
<td>% saturation (%)</td>
<td>78.9</td>
<td>83.3</td>
<td>81.7</td>
<td>83.3</td>
<td>80.7</td>
<td>82.2</td>
<td>85.6</td>
<td>85.8</td>
<td>85.9</td>
</tr>
<tr>
<td></td>
<td>S.E.M.</td>
<td>7.9</td>
<td>3.0</td>
<td>5.0</td>
<td>2.5</td>
<td>5.0</td>
<td>4.4</td>
<td>3.0</td>
<td>2.3</td>
<td>2.9</td>
</tr>
<tr>
<td>P value (&lt;)</td>
<td>0.005</td>
<td>0.02</td>
<td>0.005</td>
<td>0.001</td>
<td>0.005</td>
<td>0.001</td>
<td>0.001</td>
<td>0.6</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

*a* Animals used for the serum samples at time 0 received no injection.

*b* Total iron binding capacity.
4.0

5.8

I-

I

::3

0 3.6

Z

0

J

o

3.4

0 J

5.2

Figure 3. Correlation between the percentage iron saturation of the serum and the growth of C. albicans in the serum expressed as the logarithm of the colony count. The line was calculated with linear regression analysis and has a slope of 0.0064 with a y-intercept of 3.1862. The circles represent the results of serum samples from mice injected i.p. with 200 μg of endotoxin, the triangles represent mice injected with 1 ml of saline and the squares represent mice that received no injection.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Iron</th>
<th>% Iron Saturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endotoxin (40)</td>
<td>0.346 a</td>
<td>0.578</td>
</tr>
<tr>
<td>p value (&lt;)</td>
<td>0.025 b</td>
<td>0.001</td>
</tr>
<tr>
<td>Saline (40)</td>
<td>0.341</td>
<td>0.435</td>
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<td>p value (&lt;)</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>All samples (84)</td>
<td>0.435</td>
<td>0.588</td>
</tr>
<tr>
<td>p value (&lt;)</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Data presented are the Spearman rank correlation coefficient for each group.

* Probability value derived from Student’s t-test.

TABLE II
Correlation between colony counts of Candida albicans and serum iron and iron saturation

It has been observed that Candida growth in serum is directly related to the percentage iron saturation (14). When the iron concentration of serum exceeds the total iron-binding capacity, or free iron is present, then vigorous growth of Candida ensues (14, 15). In addition, a recent study has demonstrated that iron-chelating iron. All groups returned to essentially baseline values for serum iron by 6-hr post-injection but fell below baseline values by 24 hr. However, these data document that the iron injected i.p. is absorbed into the blood and define the magnitude and duration of the serum iron elevation.

DISCUSSION

It has been observed that Candida growth in serum is directly related to the percentage iron saturation (14). When the iron concentration of serum exceeds the total iron-binding capacity, or free iron is present, then vigorous growth of Candida ensues (14, 15). In addition, a recent study has demonstrated that iron-chelating
Figure 4. The percentage cumulative mortality of control and endotoxin-treated mice after challenge with C. albicans and injection of one of four concentrations of ferric ammonium citrate or ammonium citrate. Each group contained 25 mice. Control animals are represented by solid symbols and endotoxin-treated mice by open symbols.

Figure 5. The effect of iron on the mortality rate of endotoxin-treated (hatched bars) and control mice (solid bars) challenged with $10^4$ C. albicans i.v. On the ordinate is shown the Wilcoxon test comparing the ammonium citrate-treated control group (designated zero) with all other groups. The value of the Wilcoxon test is expressed as a standard normal deviate, that is, as (normal variate minus expected mean)/standard deviation. On the abscissa is the quantity of iron as ferric ammonium citrate given to the endotoxin-treated and non-endotoxin-treated (control) groups at the time of challenge with C. albicans. The two groups that did not receive iron were injected with ammonium citrate (806 µg) at the time of challenge. A positive standard normal deviate represents protection, while a negative standard normal deviate represents increased lethality, with levels of significance shown by the horizontal lines and the p values on the right.
TABLE III
Changes in serum percentage iron saturation after i.p. injection of ferric ammonium citrate or ammonium citrate with time

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Material Injected</th>
<th>Ammonium citrate</th>
<th>Ferric ammonium citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>806 μg</td>
<td>77.15 ± 4.76 (16)*</td>
<td>77.15 ± 4.76 (16)</td>
</tr>
<tr>
<td>1</td>
<td>50 μg Fe</td>
<td>84.08 ± 4.08 (4)</td>
<td>93.52 ± 1.07 (4)</td>
</tr>
<tr>
<td>2</td>
<td>100 μg Fe</td>
<td>96.79 ± 2.20 (4)</td>
<td>92.71 ± 2.21 (4)</td>
</tr>
<tr>
<td>4</td>
<td>150 μg Fe</td>
<td>80.60 ± 3.57 (4)</td>
<td>87.02 ± 3.47 (4)</td>
</tr>
<tr>
<td>6</td>
<td>200 μg Fe</td>
<td>79.48 ± 3.35 (4)</td>
<td>77.51 ± 4.85 (4)</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>44.22 ± 2.68 (4)</td>
<td>49.25 ± 2.80 (4)</td>
</tr>
</tbody>
</table>

*The 806 μg of ammonium citrate are the amount of ammonium citrate the mouse receives when injected with 200 μg of iron as ferric ammonium citrate.

Iron saturation to control values by the addition of iron to serum collected 24 hr post-endotoxin removed the growth-inhibiting properties of this sera. Therefore, changes in serum iron concentration seem to account for the altered growth rate of Candida in serum from endotoxin-treated mice.

A cyclical response of mice to challenge with C. albicans at increasing time intervals after endotoxin administration has been documented. Kimball et al. found a significant increase in the length of survival of mice challenged with C. albicans at 1 day and 4 through 8 days after endotoxin administration (5). A similar study by Hasenclever and Mitchell showed a maximum increase in survival 1 day after endotoxin, a return to essentially control animal survival rates on days 2 and 3, but an increase in survival on the 4th and 6th days after endotoxin (24). The consistent cyclical pattern of these two studies is strikingly similar to the changes in iron and growth of C. albicans in mouse sera after in vivo endotoxin administration in the present study (Fig. 1 and Table I). This is suggestive evidence that the resistance to Candida infection induced by endotoxin is due to changes in iron metabolism which secondarily controls the rate of growth of Candida.
hr after injection (16). The observations were not extended beyond 48 hr when the serum iron was still diminished but approaching control values. The results of the present study in mice are in agreement with these findings and extend them to show a cyclical pattern of the serum iron beyond 48 hr. Furthermore, another study reported that the total iron binding capacity (TIBC) after endotoxin injection decreased at 1 day with a return to essentially control values between the 2nd and 3rd days (25). The study was not extended beyond 72 hr. The observations were in agreement with these findings and extended them to show a cyclical pattern of the serum iron and transferrin metabolism that may last 8 days.

The increased resistance to challenge with *C. albicans* by mice 24 hr after endotoxin administration was negated and reversed by the i.p. injection of increasing quantities of iron (Figs. 4, 5). The step-like pattern of this dose-response experiment clearly demonstrates the in *vivo* importance of iron in *C. albicans* infections. Also, only those animals receiving 200 μg of iron exceeded their TIBC, thereby having free iron, for a maximum duration of 4 hr after iron injection (Table III). These findings indicate that free iron was not essential to permit an increase in the growth rate of *C. albicans in vivo* and suggest that an increase in the percentage iron saturation of the serum and an increase in the total body iron (tissue iron) enabled the *C. albicans* to multiply more rapidly. This contention is supported by the lower serum iron values 24 hr after the injection of ferric ammonium citrate or ammonium citrate (Table III).

An ever enlarging literature suggests that iron concentration in synthetic media and percentage iron saturation in body fluids are major factors affecting the growth of a wide variety of microorganisms. A review of the effect of metallic ions on host-pathogen interactions concluded that “in the contest between the establishment of bacterial or mycotic disease and the successful suppression of the disease by animal hosts, iron is the metal whose concentration in host fluids appears to be most important” (26). In the present study, changes in iron metabolism secondary to endotoxin and iron administration appear to mediate the susceptibility of animals to challenge with *C. albicans* and suggest that the nonspecific resistance to infection with a spectrum of microorganisms induced by endotoxin may also be related to these changes in iron metabolism.

REFERENCES