INFECTION AND IMMUNITY, Feb. 1983, p. 659–665
0019-9567/83/020659-07$02.00/0

Vol. 39, No. 2

Acute Iron Overload in Mice: Pathogenesis of *Salmonella typhimurium* Infection

GÜNTHER SAWATZKI,* FRANZ A. HOFFMANN, AND BERNHARD KUBANEK

Department of Transfusion Medicine, University of Ulm, D-7900 Ulm, Federal Republic of Germany

Received 3 June 1982/Accepted 28 October 1982

The bacterial growth in the tissues of C3D2F1 male mice was measured during an experimental infection with two *Salmonella typhimurium* strains (high virulence, strain 2386/74; low virulence, strain L15403). This experimental model was used for evaluation of the pathogenesis in normal and iron-overloaded animals. Acute iron overload was accomplished by intramuscular injections of chelated iron (with 2,3-dihydroxybenzoic acid and citrate) with a single dose of 100 μg of iron per mouse. Bacteria were given intraperitoneally 1 h after the iron injection. Serum iron levels, transferrin levels, and the bacteria counts in blood and liver were measured simultaneously in all animals. There was a significant increase of bacterial growth in all tissues in the iron-treated animals. Iron abolished the normal clearance of the bacteria with low virulence from the blood. This study demonstrates that a general iron overload, as determined by an increased serum iron level, resulting from preinjection of iron, enhances bacterial growth.

Normal serum inhibits bacterial growth by different factors. In 1946, Schade and Caroline were able to show that the addition of iron can partially abolish this bacteriostasis of serum (23). The normal bacteriostatic effect was interpreted as a result of the very small amounts of free iron available in the serum due to the iron-binding proteins compared with the absolute requirement of iron for bacterial growth (26). These experiments implicated the iron-binding proteins, transferrin and lactoferrin, as key molecules involved in the nonspecific host resistance (1). Many investigators have tried to elucidate by in vivo and in vitro experiments the molecular basis for this effect. Some observations implied that the iron-binding proteins could inhibit bacterial growth in vitro (1, 12). It was suggested that the virulence of potential pathogens was associated with the ability of the bacteria to acquire iron from the host by producing siderophores (5, 21, 28). The importance of a plasmid as a factor for virulence was discussed by others (3, 25). Most in vivo experiments were performed with iron-overloaded animals. Whereas some authors only monitored increased mortality in these animals after an experimental infection (10, 11, 15, 17, 19), others were able to show an enhancing effect of iron on the bacterial counts in the infected animals without measuring the actual serum iron level in relation to the bacterial growth in the same animals (2, 4, 7–9, 16, 24). In addition to the serum iron level, the transferrin level should be measured, since the saturation of this iron-binding protein with iron is thought to be an important factor for the availability of iron to the bacteria (17). All these requirements were realized only partially in very few studies (8, 16, 24). Another aspect which must not be overlooked is that iron overload must be present not as a local iron overload but as a systemic iron overload. A local iron overload at the same site of the bacterial injection may reflect an isolated enhancement of bacterial growth rather than an inhibition of a physiological defense mechanism of the host.

The experiments described in this paper deal with an acute iron overload in mice and its effect on bacterial growth in liver and blood after an infection with two strains of *Salmonella typhimurium* of different virulence. The iron and transferrin levels in the blood were measured consecutively. The correlation between these parameters of serum iron status and bacterial growth supports the hypothesis that serum iron status is an important factor in the host defense mechanism against bacterial infection.

MATERIALS AND METHODS

Bacteria strains. *S. typhimurium* L15403, with a 50% lethal dose of 5×10^5 for male mice, was supplied by F. Kayser, Mikrobiologisches Institut, Zürich, Switzerland, and a highly virulent strain of *S. typhimurium*, 2386/74, with a 50% lethal dose of 100 for male mice, was from the Institut für Veterinärmedizin des Bundesgesundheitsamtes, Berlin, West Germany.

Mice. Male specific-pathogen-free C3H/Tif × DBA/2/Bom F1 hybrid (C3D2F1) from Gl. Bomholtgard Ltd., Ry, Denmark, between 8 and 10 weeks of

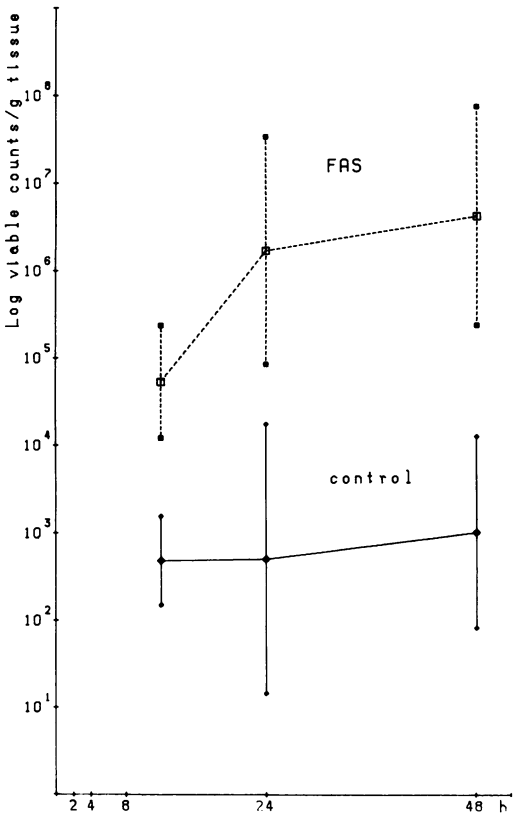

FIG. 1. Effect of i.p. injection of 100 μg of iron as FAS on the growth of an avirulent strain of *S. typhimurium* L15403 (inoculum, 1.9×10^5) in the liver. Symbols: ◇, *S. typhimurium* L15403; □, *S. typhimurium* L15403 plus 100 μg of Fe as FAS.

age, were used. Five randomly selected mice were kept in plastic cages and were given food and water ad libitum.

Infection studies. Mice were injected intraperitoneally (i.p.) with *S. typhimurium* (grown on DST agar [Oxoid no. CM 261]) suspended in 0.5 ml of 0.9% NaCl solution.

The inocula were: experiment 1, 1.9×10^5 *S. typhimurium* L15403; experiment 2, 9.6×10^2 *S. typhimurium* L15403; experiment 3, 9.0×10^2 *S. typhimurium* L15403 and 1.7×10^3 *S. typhimurium* 2386/74. The controls were injected only with bacteria. Bacterial enumeration in blood and liver was determined as described previously (14). Five mice of each group were killed by cervical dislocation at each time point. Blood was obtained by cardiac puncture. For the determination of bacteria in blood and in liver homogenates, 10-fold serial dilutions were performed. From each dilution, 1 ml was mixed with 15 ml of DST agar in petri dishes. After incubation (37°C for 24 h), all CFU were counted.

Iron compounds. In experiment 1, ferric ammonium sulfate (FAS) was injected i.p. A 100-μg amount of Fe (1.73 mg of FAS per ml) was given in a volume of 0.5 ml. In experiment 2, 100 μg of Fe (4.32 mg of FAS per ml) complexed with citrate (5.27 mg of $C_6H_5Na_3O_7 \cdot 2H_2O$ per ml) was injected in a volume of 0.2 ml intramuscularly (i.m.). In experiment 3, 100 μg of Fe (4.32 mg of FAS per ml) complexed with 2,3-dihydroybenzoic acid (2,3-DHB) (1.38 mg of 2,3-DHB per ml) was injected i.m. in a volume of 0.2 ml. As a control, a 2,3-DHB solution (1.38 mg of 2,3-DHB per ml) was used.

Medium. Bacteria were grown on DST agar for 24 h at 37°C.

Iron status. Serum iron was determined colorimetrically by the ferrozine method (6). Transferrin levels in the serum were determined by rocket immunoelectrophoresis (22). Both parameters were determined for each animal.

All experiments were started at 8 a.m. with the iron injection. One hour later, the animals were challenged with the bacteria (with the exception of experiment 1, in which a 2-h delay was chosen). For bacterial enumeration and determination of iron status, five animals of each group were killed after 12, 24, and 48 h (experiment 1) or after 2, 8, 24, and 48 h (experiments 2 and 3). Means and standard deviations are reported. Statistical analysis was done with the Student *t* test.

RESULTS

Influence of FAS injected i.p. In this experiment, iron as FAS was injected i.p. 2 h before the inoculation of bacteria (*S. typhimurium* L15403). Bacterial counts were measured at 12, 24, and 48 h after inoculation in liver (Fig. 1). After 24 h, all bacterial counts in the tissues (log viable counts) were significantly ($P < 0.001$) increased in the iron-treated animals. In another experiment with FAS injected i.m., no such differences were detectable (data not shown). This prompted us to use chelated iron, because of the assumption that these iron complexes would be better distributed through the whole body in the critical time period.

Influence of i.m. injection of citrate- and 2,3-DHB-chelated iron. Iron was injected i.m. (100 μg of Fe) as ferric citrate or ferric 2,3-DHB. One hour later, the bacteria (*S. typhimurium* L15403) were injected i.p. At 2, 8, 24, and 48 h after this challenge, the bacterial counts in blood were determined and correlated to the serum iron levels at the same time points (Fig. 2A).

The increased serum iron level at 2 h in the Fe–2,3-DHB-treated group was followed by an increase of the bacterial counts in blood after 8 h (Fig. 2A). Bacterial counts in liver (Fig. 2B) showed a significant ($P < 0.05$) enhancing effect of the ferric 2,3-DHB complex beginning at 24 h after inoculation, whereas there was no significant effect of the ferric citrate at 24 h. Bacterial counts were still elevated in the blood 24 h after injection of the 2,3-DHB iron complex as compared to the control (Fig. 2A). At 24 h, there was a slight but not significant decrease in serum iron in the ferric 2,3-DHB complex-treated group

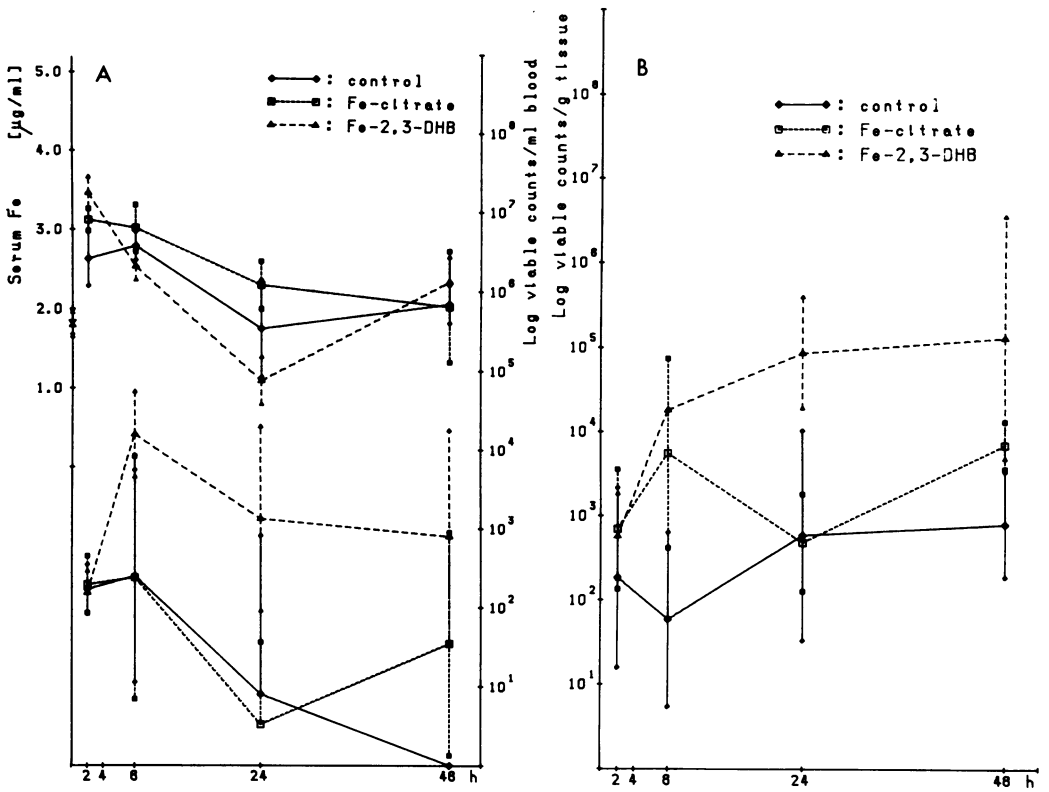

FIG. 2. Effect of i.p. injection of 100 µg of iron as Fe-citrate or as Fe-2,3-DHB on the growth of *S. typhimurium* L15403 (inoculum, 960) in the blood (A; lower diagram) and in the liver (B) and effect on serum iron level (A, upper diagram) in male C3D2F1 mice.

compared to the control (Fig. 2A). This is probably due to the hypoferremic effect of bacterial infections (26), which was seen in most of our experiments at high bacterial counts.

The more pronounced effect of the 2,3-DHB complexed iron compared to the citrate complex led us to consider whether 2,3-DHB alone (as the precursor of enterobactin synthesis) could cause the same effect.

Comparison of the ferric 2,3-DHB complex and 2,3-DHB alone with both strains of *S. typhimurium*. The experimental design was the same as that in the experiment described above, but as potential enhancing factors, ferric 2,3-DHB complex and iron-free 2,3-DHB were tested.

This experiment was repeated with the highly virulent strain *S. typhimurium* 2386 to test the effect of iron overload on bacterial strains of differing virulence. The viable counts in liver for the strain with low virulence (*S. typhimurium* L15403) are shown in Fig. 3, and data for the highly virulent strain (*S. typhimurium* 2386) are shown in Fig. 4. Compared with the control, the iron-treated group had significantly increased numbers of bacterial counts in liver for the bacterial strains of differing virulence (*S. typhimurium* L15403, $P < 0.025$; *S. typhimurium* 2386, $P < 0.01$) from 24 h on. The injection of iron-free 2,3-DHB had no enhancing effect for either bacterial strain in the liver. The serum iron levels together with the viable bacterial counts in blood for both experiments are shown in Fig. 5. Transferrin levels were measured (Fig. 6). There were no significant changes in transferrin levels between the differently treated groups challenged with *S. typhimurium* L15403 (low virulence) for 48 h. In contrast, the groups challenged with the highly virulent *S. typhimurium* 2386 showed differing levels of serum transferrin at 48 h. The animals challenged with the highly virulent strain (*S. typhimurium* 2386) and the iron complex showed no hypoferremia (Fig. 5B), in contrast to the animals challenged with the *S. typhimurium* L15403 (low virulence) (Fig. 5A).

There is another effect worth noting: during infection with the highly virulent strain (*S. typhimurium* 2386) without iron injection, the bacteria were not completely cleared from the blood (Fig. 5B) until 48 h, whereas there was complete clearance from blood (Fig. 5A) of the strain with low virulence at 48 h.

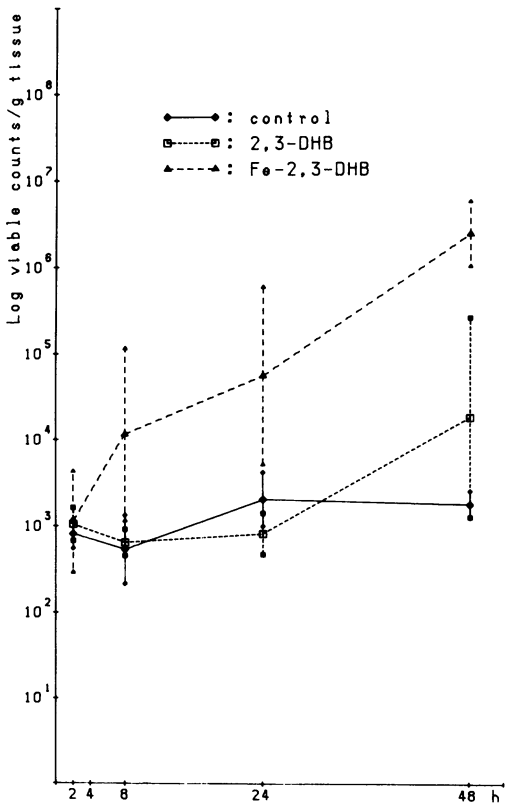

FIG. 3. Effect of i.m. injection of 2,3-DHB and 100 μg of iron as Fe-2,3-DHB on the growth of *S. typhimurium* L15403 (inoculum, 900) in the liver.

DISCUSSION

The enhancement of bacterial counts after injection of bacteria and iron at the same site (i.p.) could be reproduced in our infection model in a manner similar to that shown by others for *Listeria monocytogenes* (24) and *Neisseria meningitidis* (8, 9) in mice and for *Pseudomonas aeruginosa* in rabbits (2). This local iron overload effect is associated with higher mortality rates reported by our group (19) and by others (11). Experiments in which iron and bacteria are injected at the same site should be interpreted with caution, since the unphysiological high local iron concentrations in the peritoneal cavity facilitate bacterial growth. Therefore, in further studies, another experimental design was used which reflects physiological conditions: i.m. injections of the iron compounds and i.p. injections of the bacteria. In this way, we could show that a systemic iron overload has a significant effect on bacterial growth. In only two reported studies (8, 24), serum iron levels were measured and showed a course of bacterial growth similar to that reported here. Sword (24) reported serum iron levels based not on individual but on pooled sera. From the study of Holbein (8), it is not clear whether the serum iron levels after iron injection were measured in infected animals. These studies are therefore not direct evidence for the hypothesis that the relative changes in serum iron to transferrin levels will predetermine the fate of the infection (23, 26). From our experiments, it can be concluded that elevated serum iron levels after injection of complexed iron promote the bacterial multiplication in blood and liver for the next 48 h and thus accelerate the infection. There are some indications that iron deficiency, which results in increased serum transferrin levels, has a protective effect against bacterial infections (20). The role of transferrin in this respect should be the subject of further studies. No changes in transferrin levels were observed during our experiments with the bacteria strain with low virulence, but there was an increase in the transferrin level in the animals treated with the highly virulent strain with either the chelator or

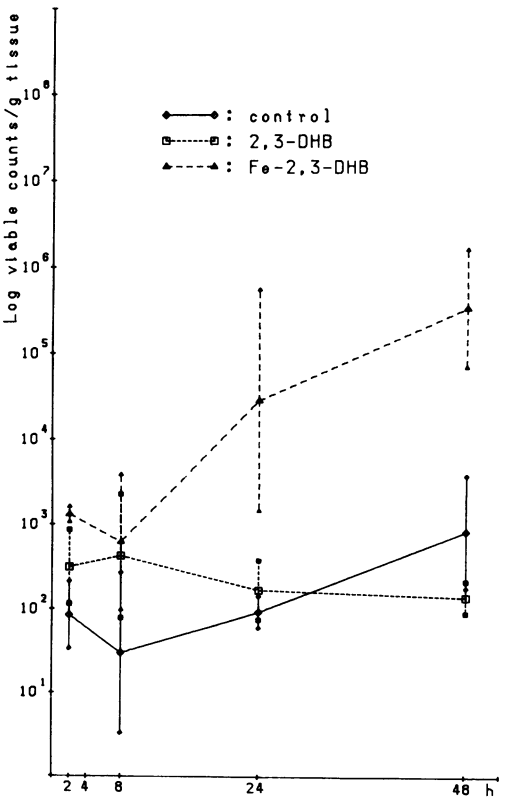

FIG. 4. Effect of i.m. injection of 2,3-DHB or 100 μg of iron as Fe-2,3-DHB on the growth of the highly virulent strain *S. typhimurium* 2386 (inoculum, 1,700) in the liver.

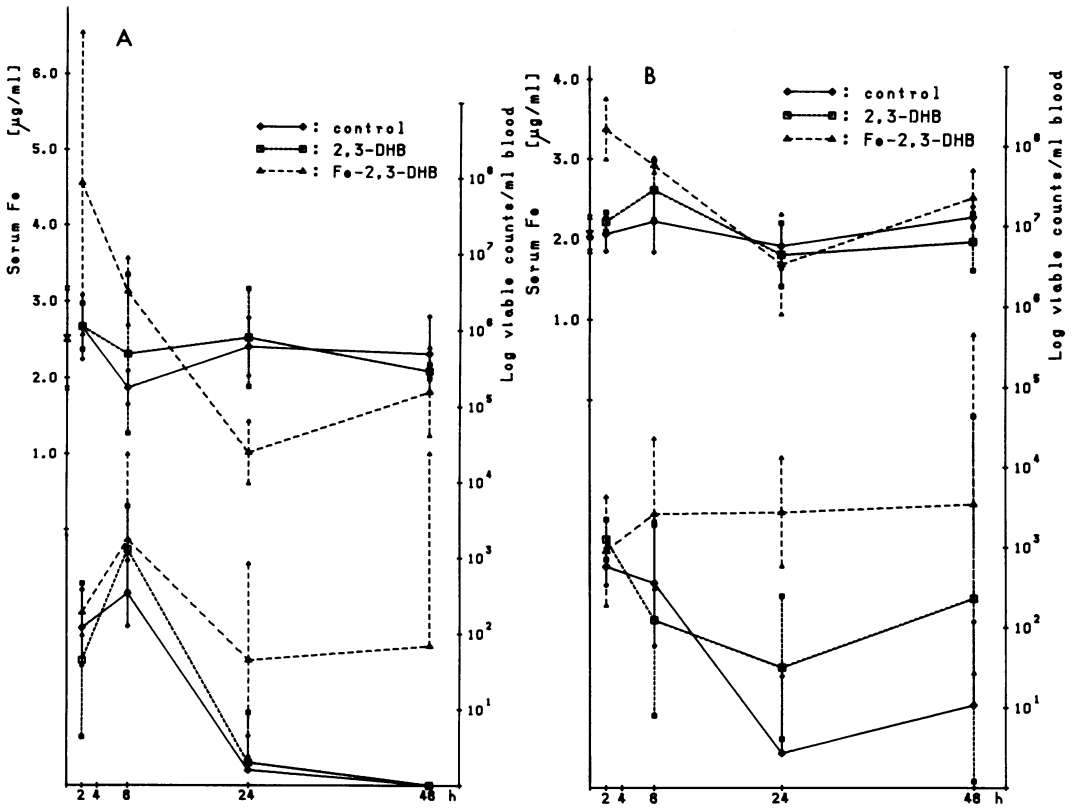

FIG. 5. (A)Effect of i.m. injection of 2,3-DHB and 100 µg of iron as Fe-2,3-DHB on the growth of *S. typhimurium* L15403 (inoculum, 900) in the blood (lower diagram) and effect on the serum iron level (upper diagram). (B) Effect of the same injections on the growth of *S. typhimurium* 2386 (inoculum 1,700) in the blood (lower diagram) and effect on the serum iron level (upper diagram).

the iron complex. Thus, the question of whether transferrin influences bacterial growth cannot be answered from these observations.

The enhanced bacterial multiplication rate under iron overload conditions was observed for bacteria with both high and low virulence. Thus, increasing the availability of iron minimized the differences of virulence in our experiments. The highly virulent bacteria can survive despite restricted availability of iron in the blood of untreated mice (Fig. 5B). The bacteria with low virulence were cleared from the blood in 48 h in normal controls. Iron overload, on the other hand, suppressed this clearance (Fig. 5A).

However, virulence is related to many other factors. This work deals only with the role of iron on the growth of *Salmonella* strains of differing virulence. Therefore, our discussion about virulence is necessarily restricted.

Our observations could suggest two hypothesis for the different virulence of the *S. typhimurium* strains used in these experiments. As a first hypothesis, we suggest that the more virulent strain has a better iron-retrieving mechanism in that it produces more enterobactin or has a better uptake system for siderophores (5, 13, 21, 28). This has been questioned by others (11). The second hypothesis is that there is a plasmid which can be expressed under low iron conditions by the highly virulent strain, resulting in the synthesis of a highly potent siderophore (25). 2,3-DHB as a complexing agent for iron was used, since this substance is the natural precursor of the siderophore enterobactin which is normally produced by *Salmonella* species. In our experiments, however, there was no enhancing effect on bacterial growth of this substance without additional iron. In another study (27), there was also no stimulation of growth of *S. typhimurium* by 2,3-DHB in serum. Others (18) have shown that there are mutants of *S. typhimurium* with blocked biosynthesis of enterobactin which cannot grow on citrate with supplementation of 2,3-DBH: class I of the *enb* mutants. We therefore assume that our strains are class I *enb* types. This suggests that the

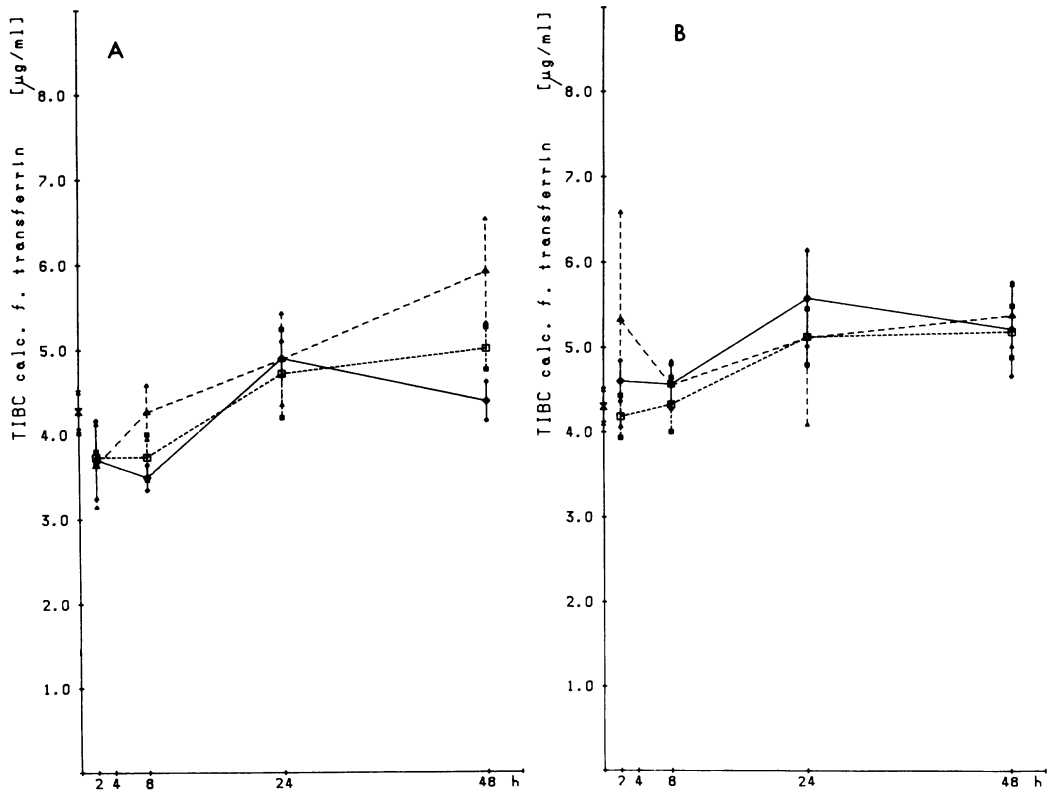

FIG. 6. Effect on 2,3-DHB and 100 µg of Fe as Fe-2,3-DHB and the injection of *S. typhimurium* 2386 (inoculum 1,700) (A) and L15403 (inoculum 900) (B) on the total iron-binding capacity. Symbols: ◇, control; □, 2,3-DHB; △, Fe-2,3-DHB.

difference of our strains in virulence does not depend on a difference in the utilization of 2,3-DHB as an iron chelator.

Further investigations are in progress using the iron-binding proteins transferrin or lactoferrin for modulating the iron status rather than iron overload.

ACKNOWLEDGMENTS

This work was supported by grant SFB 112/E7 from the Deutsche Forschungsgemeinschaft.

The authors acknowledge the excellent technical assistance of G. Hruby and M. Wich and the help of P. Byrne in discussing and reading the manuscript.

LITERATURE CITED

1. **Bullen, J. J., H. J. Rogers, and E. Griffiths.** 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. **80**:1–35.
2. **Bullen, J. J., C. G. Ward, and S. N. Wallis.** 1974. Virulence and the role of iron in *Pseudomonas aeruginosa* infection. Infect. Immun. **10**:443–450.
3. **Crosa, J. H.** 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. Nature (London) **284**:566–568.
4. **Fletcher, J., and E. Goldstein.** 1970. The effect of parenteral iron preparations on experimental pyelonephritis. Br. J. Exp. Pathol. **51**:280–285.
5. **Griffiths, E., and J. Humphreys.** 1980. Isolation of enterochelin from the peritoneal washings of guinea pigs lethally infected with *Escherichia coli*. Infect. Immun. **28**:286–289.
6. **Harris, D. C.** 1978. Serum iron determination: a sensitive colorimetric experiment. J. Chem. Educ. **55**:539–540.
7. **Henderson, L. C., S. Kadis, and W. L. Chapman, Jr.** 1978. Influence of iron on *Corynebacterium renale*-induced pyelonephritis in a rat experimental model. Infect. Immun. **21**:540–545.
8. **Holbein, B. E.** 1980. Iron-controlled infection with *Neisseria meningitidis* in mice. Infect. Immun. **29**:886–891.
9. **Holbein, B. E.** 1981. Enhancement of *Neisseria meningitidis* infection in mice by addition of iron bound to transferrin. Infect. Immun. **34**:120–125.
10. **Jones, R. L., C. M. Peterson, R. W. Grady, T. Kumbaraci, A. Cerami, and J. H. Graziano.** 1977. Effects of iron chelators and iron overload on *Salmonella* infection. Nature (London) **267**:63–65.
11. **Kochan, I., J. Wasynczuk, and M. A. McCabe.** 1978. Effects of injected iron and siderophores on infections in normal and immune mice. Infect. Immun. **22**:560–567.
12. **Kvach, J. T., T. I. Wiles, M. W. Mellencamp, and I. Kochan.** 1977. Use of transferrin-iron-enterobactin complexes as the source of iron by serum-exposed bacteria. Infect. Immun. **18**:439–445.
13. **Leong, J., and J. B. Neilands.** 1976. Mechanisms of siderophore iron transport in enteric bacteria. J. Bacteriol. **126**:823–830.
14. **Mackaness, G. B., R. V. Blanden, and F. M. Collins.** 1966. Host-parasite relations in mouse typhoid. J. Exp. Med. **124**:573–583.
15. **Miles, A. A., P. L. Khimji, and J. Maskell.** 1979. The

variable response of bacteria to excess ferric iron in host tissues. J. Med. Microbiol. **12**:17–28.
16. **Payne, S. M., and R. A. Finkelstein.** 1978. The critical role of iron in host-bacterial interactions. J. Clin. Invest. **61**:1428–1440.
17. **Polk, H. C., Jr., and A. A. Miles.** 1971. Enhancement of bacterial infection by ferric iron: kinetics, mechanisms, and surgical signficance. Surgery **70**:71–77.
18. **Pollack, J. R., B. N. Ames, and J. B. Neilands.** 1970. Iron transport in *Salmonella typhimurium*: mutants blocked in the biosynthesis of enterobactin. J. Bacteriol. **104**:635–639.
19. **Puschmann, M., and A. M. Ganzoni.** 1977. Eisenueberladung: einfluss auf spezifische und unspezifische Immunitaet. Schweiz. Med. Wochenschr. **107**:1774–1776.
20. **Puschmann, M., and A. M. Ganzoni.** 1977. Increased resistance of iron-deficient mice to *Salmonella* infection. Infect. Immun. **17**:663–664.
21. **Rogers, H. J.** 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immun. **7**:445–456.
22. **Sawatzki, G., V. Anselstetter, and B. Kubanek.** 1981. Isolation of mouse transferrin using salting-out chromatography on Sepharose CL-6B. Biochim. Biophys. Acta **667**:132–138.
23. **Schade, A. L., and L. Caroline.** 1946. An iron-binding component in human blood plasma. Science **104**:340–341.
24. **Sword, C. P.** 1966. Mechanisms of pathogenesis in *Listeria monocytogenes* infection. I. Influence of iron. J. Bacteriol. **92**:536–542.
25. **Warner, P. J., P. H. Williams, A. Bindereif, and J. B. Neilands.** 1981. ColV plasmid-specified aerobactin synthesis by invasive strains of *Escherichia coli*. Infect. Immun. **33**:540–545.
26. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.
27. **Wilkins, T. D., and C. E. Lankford.** 1970. Production by *Salmonella typhimurium* of 2,3-dihydroxybenzoylserine, and its stimulation of growth in human serum. J. Infect. Dis. **121**:129–136.
28. **Yancey, R. J., S. A. L. Breeding, and C. E. Lankford.** 1979. Enterochelin (enterobactin): virulence factor for *Salmonella typhimurium*. Infect. Immun. **24**:174–180.