LOW MOLECULAR WEIGHT IRON-BINDING FACTOR
FROM MAMMALIAN TISSUE
THAT POTENTIATES BACTERIAL GROWTH

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Over 20 years ago, Schade and Caroline (1) found that an iron-binding protein in egg white was bacteriostatic. Since then it has become clear that the competition between host and pathogen for trace nutrients, particularly iron, can be an important determinant to the virulence of an invading microorganism. For example, during acute infections in mammals, plasma iron is shifted toward the reticuloendothelial system, presumably making it less available to invading pathogens (2). Furthermore, iron-binding proteins such as transferrin in the plasma and lactoferrin in secretions have been shown to be bacteriostatic, both in vitro and in vivo (3). Many investigations have shown that sequestration of iron may be an important initial step in the host’s defenses against infectious disease (4).

On the other hand, many microorganisms are able to produce their own iron-binding molecules when faced with a low-iron environment (5). These compounds have iron-binding constants that are comparable to those of transferrin and lactoferrin (6) and would appear to be the pathogen’s answer to the host’s iron-binding defense mechanisms. Indeed, the ability to produce these compounds in vitro is associated with increased virulence of many bacteria in vivo (7). However, these compounds have not been isolated from an infected host. And, although the inability to produce these compounds may be associated with reduced virulence, pathogens unable to synthesize such compounds are fully capable of producing disease (8). Thus, organisms must be able to obtain iron for growth by another mechanism.

This report describes the partial purification and characterization of a low molecular weight iron-binding compound isolated from mammalian tissue capable of supplying iron to, and stimulating the growth of, several pathogenic bacteria. This host-associated iron transfer factor (HAITF) may play an important role in the competition between host and pathogen for iron.

Materials and Methods

Reagents. Chromatographic resins AG-50W-X8 and Chelex-100 were purchased from Bio-Rad Laboratories, Richmond, Calif. Sephadex G-10, G-15, G-25, and G-50 gel filtration beads were purchased from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J. Desferrioxamine B was obtained from Ciba Pharmaceutical Co., Div. of Ciba-Geigy Corp., Summit, N. J.

Bacteria. Salmonella typhimurium mutants TA 2701 and TA 2700 were obtained from Dr.
Hugh Akers and Dr. M. Luckey (9). Wild-type pathogenic S. typhimurium, Escherichia coli, and Klebsiella pneumoniae were obtained from isolates from patients with clinical infectious disease.

**Assay for HAITF.** Minimal medium E of Vogel and Bonner (10) in 1.5% agar was used as low-iron medium. After the medium was prepared, autoclaved, and cooled to 45°C, a saline-washed suspension of S. typhimurium TA 2700 was added, and the resultant mixture was poured into 100 × 15-mm Petri dishes. S. typhimurium TA 2700 is an enb mutant unable to synthesize the iron chelator produced by wild-type salmonellae enterobactin, and thus is unable to grow on low-iron medium unless supplied with an exogenous iron chelator.

HAITF activity was assayed by drying 5, 10, or 20 µl of sample on a 6-mm filter paper disk. The disks were placed on the prepared plates and incubated at 37°C for 24 h. The amount of activity was determined by recording the mean of three measurements of the distance between the edge of the disk and the edge of the halo of growth.

**Amount of HAITF in Various Tissues.** Five male Sprague-Dawley rats were sacrificed by decapitation. The blood was collected and clotted. Each animal was then dissected and samples of various organs were obtained. The tissue was washed free of loose blood, patted dry, and then weighed. The tissue was then homogenized with 9 vol of anhydrous methanol in a Virtis homogenizer (VirTis Co., Inc., Gardiner, N. Y.). After the homogenate was centrifuged, the supernate was decanted and dried overnight in a desiccator. The dried extract was redissolved in 1 vol of 0.025 M ammonium acetate. Duplicate aliquots (20 µl) of this solution were assayed for bioactivity. Blood was analyzed by adding 1.0 ml of serum dropwise to 9.0 ml of methanol. After centrifugation, the supernate was dried and redissolved in 1.0 ml, 0.025 M ammonium acetate. Aliquots were assayed in duplicate as above.

**Purification of HAITF.** Horse liver was obtained from a local abattoir and washed free of blood with cold-distilled water. Unless stated otherwise, all steps were performed at 4°C. The liver was homogenized at high speed in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) for 20 min with an equal volume of glass-distilled water. The homogenate was centrifuged at 3,000 rpm for 30 min in a Sorvall RC-5 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The supernate was subjected to ultrafiltration through dialysis tubing and the filtrate recovered. The filtrate was dried by flash evaporation and the residue extracted with anhydrous methanol. Methanol-insoluble material was removed by filtration. The methanol-soluble extract was lyophilized and stored at -20°C for up to 8 mo without loss of activity.

2 g of methanol-soluble extract dissolved in 10 ml of 0.1 M NaCl was loaded onto a 2.6 × 32-cm column that contained AG 50W-X8 resin (hydrogen form) that had been equilibrated with at least 1 liter of distilled H2O. The column was eluted at room temperature with a 0.1-0.5 M NaCl gradient and then with 1.0 M NaCl. Fractions that contained HAITF bioactivity were pooled, dried by rotary evaporation, and the residue extracted several times with methanol. To remove iron from the sample, the methanol-soluble material from the AG-50 column was dried by rotary evaporation, dissolved in 10 ml of glass-distilled water, and eluted over a column (1.0 × 18 cm) of Chelex-100 resin that had been previously washed with 1 M sodium acetate and equilibrated with glass-distilled water. The iron concentration of eluted fractions was measured with a Perkin-Elmer atomic absorption spectrophotometer (model 305B, Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). The iron-free sample of HAITF was then dissolved in 10 ml of 0.025 M ammonium acetate and applied and eluted from a Sephadex G-50 column (3.0 × 100 cm) previously equilibrated with 0.025 M ammonium acetate.

**Assessment of Iron Binding by HAITF.** Samples of HAITF were incubated with 1 µCi of 59FeCl3 (dissolved in 0.1 µl HCl, 13.77 µCi/mg sp act) and applied as 1- to 2-mm spots to Whatman 3M chromatography paper (Whatman, Inc., Clifton, N. J.). Samples of HAITF or 59FeCl3 alone were also spotted as controls. Ascending chromatography was performed with propanol-water (70:30 vol/vol). The various lanes of the chromatograms were either sprayed with 1% FeCl3 solution to test for iron staining, cut into segments and counted in a Packard gamma counter (model 575, Packard Instrument Co., Inc., Downers Grove, Ill.), or disks (6 mm) were punched out and assayed for bioactivity.

Samples of HAITF incubated with 59FeCl3 were also applied to Sephadex G-10, G-15, G-25, or G-50 columns. The columns were eluted at 4°C with 0.025 M ammonium acetate buffer, and the resulting fractions were counted for 59Fe radioactivity and assayed for bioactivity.

**Iron-Uptake Experiments.** Iron-uptake experiments were performed by the method of Peters.
and Warren (11). These experiments were carried out with resting cells grown to a density of $10^9$ bacteria/ml in low-iron medium. 15-ml suspensions were placed in 25-ml Erlenmeyer flasks. $^{59}$FeCl$_3$ in saline solution was added to each flask either with or without added HAITF. Suspensions were agitated in a shaking water bath at 37°C. Aliquots were withdrawn at intervals and filtered through 0.45-µm Millipore filters (Millipore Corp., Bedford, Mass.). The filters were flushed with low-iron medium and counted in a gamma counter for retained radioactivity.

**Bacterial Growth-Curve Experiments.** Organisms were grown overnight in nutrient broth and washed three times with sterile saline. Aliquots (20 µl) of a suspension that contained $10^9$ organisms/ml were added to 1 ml of sterilized (0.45 µm Millipore filter) liquid minimal medium to which had been added a solution of HAITF or sterile saline. Growth was followed spectrophotometrically at 450 nm in a Coleman spectrophotometer (Coleman Systems, Irvine, Calif.).

**Effect on HAITF on Bacterial Infection In Vivo.** Male 8- to 10-wk-old BALB/c mice were infected with wild-type *S. typhimurium* (Fig. 7 A) or mutant *S. typhimurium* TA 2701 (Fig. 7 B) by an intraperitoneal injection. At the time of infection, they began receiving subcutaneous injections of a sterilized solution of HAITF. Injections of 0.1 ml HAITF (850 U/ml) were given every 6 h and continued until six injections were received by each mouse. The number of survivors in each group of mice was recorded every 6 h. Results were analyzed statistically by the Stromgen method of life-table analysis (12).

**Results**

**Assay for HAITF.** HAITF bioactivity was quantitated by measuring the distance between the edge of a filter paper disk saturated with sample and the outer edge of a halo of bacteria growing around the disk. Fig. 1 shows the response (in millimeters of growth) after 24 h of incubation with varying amounts of HAITF. A unit is defined as 1 mm of growth of TA 2700 in 24 h at 37°C.

**Amount of HAITF in Various Tissues.** The methanol-extractable bioactivity of various tissues of the rat is seen in Table I. The results are expressed as units per gram tissue. The liver is the tissue with the highest amounts of methanol-extractable bioactivity and the brain is the lowest. All tissues and blood serum exhibited bioactivity.

**Purification.** The purification scheme together with the degree of purification achieved is shown in Fig. 2. Horse liver homogenized with distilled water had a 0.51 U/mg sp act. Centrifugation, ultrafiltration, and methanol extraction increased the

![Fig. 1. Relationship of amount of HAITF sample placed on filter paper disk for bioassay to bioactivity. Amount is represented by microliters of sample and bioactivity by units (one unit = 1 mm of growth in 24 h).](image)
TABLE I
Levels of HAITF in Extracts of Various Organs of the Rat

| Rat | Blood | Brain | Spleen | Liver | Heart | Kidney | Bowel | Lung |
|-----|-------|-------|--------|-------|-------|--------|-------|------|
| 1   | 800   | 800   | 875    | 990   | 850   | 910    | 1,015 | 700  |
| 2   | 650   | 765   | 840    | 1,015 | 950   | 940    | 715   | 790  |
| 3   | 650   | 790   | 1,090  | 975   | 765   | 965    | 890   | 790  |
| 4   | 675   | 765   | 865    | 1,115 | 740   | 875    | 850   | 815  |
| 5   | 625   | 765   | 925    | 1,090 | 740   | 965    | 790   | 825  |
| Mean ±1 SD | 680 ± 69.4 | 777 ± 16.8 | 819 ± 100.5 | 1,037 ± 62.1 | 826 ± 82.3 | 951 ± 38.6 | 854 ± 112.1 | 784 ± 49.4 |

Horse Liver Homogenate (SA = 0.51 units/mg)
   - dialysis, methanol extraction
   - Crude Extract (SA = 5.37 units/mg)
   - Cation-exchange chromatography
   - Activity with Iron Peak (SA = 15.8 units/mg, Fe = 0.189 µg/mg)
   - Chelex column
   - Post Chelex Activity (SA = 14.0 units/mg, Fe = 0.003 µg/mg)
   - Sephadex G-50 column
   - Post G-50 Activity (SA = 28.0 units/mg)

Fig. 2. Purification scheme of HAITF. SA = specific activity in units per milligram of dry sample.

Specific activity to 5.37 U/mg. Cation-exchange chromatography on AG 50-X8 resin resulted in the HAITF activity being eluted with 1 M salt. This solution contained a large amount of iron as measured by atomic absorption spectrometry (0.189 mg of iron/mg of sample). To dissociate HAITF activity from that of inorganic iron, the sample was passed through an iron-binding resin, Chelex-100, (sodium form) at pH 5.6. The specific HAITF activity of the sample after iron removal was 14 U/mg, whereas the iron content was reduced to 0.003 µg/mg.

The sample was evaporated to dryness and redissolved in 0.025 M ammonium acetate buffer and placed on a column of Sephadex G-50. The specific activity of the peak of activity eluted from this column was 28 U/mg. The overall purification from horse liver homogenate was ~54-fold.

Iron-Binding Assays. Samples of HAITF incubated in the presence or absence of $^{59}$FeCl$_3$ were applied to paper chromatograms as was $^{59}$FeCl$_3$ alone. After ascending chromatography, a lane that contained HAITF incubated with $^{59}$FeCl$_3$ and a lane of $^{59}$FeCl$_3$ alone were cut into 5-mm segments and counted for radioactivity. HAITF bioactivity was assayed by cutting 6-mm disks from lanes that contained HAITF, HAITF with $^{59}$FeCl$_3$, and $^{59}$FeCl$_3$ alone. A peak of radioactivity was found to move from the origin with an R$_f$ of 0.61 in the lane with HAITF incubated with $^{59}$FeCl$_3$. No migration of radioactivity was seen in the lane with $^{59}$FeCl$_3$ alone. HAITF bioactivity was not seen in the lane with $^{59}$FeCl$_3$ alone but was seen as a broad peak in those lanes that contained HAITF and HAITF incubated with $^{59}$FeCl$_3$. Lanes that con-
tained HAITF and HAITF plus $^{59}$FeCl$_3$ were sprayed with 1% FeCl$_3$. Both of these lanes showed pink staining areas with an $R_f$ of 0.55.

Samples of HAITF that had been incubated with $^{59}$FeCl$_3$ were eluted over Sephadex G-10, G-15, G-25, and G-50 columns. Both bioactivity and radioactivity were found in the exclusion volume of G-10 and G-15 columns (Fig. 3). Radioactivity and bioactivity were retained and coeluted from G-25 ($[Ve/Vo] = 1.23$) and G-50 ($[Ve/Vo] = 1.9$) columns.

Iron Uptake by Bacteria. The effect of HAITF activity on iron uptake by wild-type S. typhimurium in a low-iron medium was determined by comparing uptake of $^{59}$FeCl$_3$ with and without added HAITF (Fig. 4 A). Iron uptake by wild-type salmonella in the presence of HAITF is increased by ~25%. To examine the specificity of the iron-uptake phenomena, the same experiments were repeated with the mutant TA 2701 (Fig. 4 B). This experiment was carried out with TA 2701 and desferrioxamine as above. Again, HAITF stimulated iron uptake, whereas the presence of 5 $\mu$M of desferrioxamine significantly inhibited the iron uptake in the presence of HAITF. The presence of desferrioxamine alone completely inhibits iron uptake by these bacteria.

Bacterial Growth Stimulation. In addition to its effect on the salmonella mutant, HAITF stimulates the growth of wild-type S. typhimurium in low-iron medium. Fig.
Fig. 4. (A) Iron uptake in wild-type S. typhimurium. Each point represents counts retained on 0.45-μm filters from aliquots of bacterial suspensions. The suspensions contained 59FeCl₃ + 200 μl saline or 200 μl of HAITF. (B) Iron uptake in S. typhimurium TA 2701. Bacterial suspensions were incubated with 59FeCl₃ and 200 μl HAITF (○), 200 μl saline (□), 200 μl HAITF + 5 μM desferrioxamine (△), or 5 μM desferrioxamine (○). Aliquots were filtered over 0.45-μm filters and retained radioactivity determined.

Fig. 5 A shows the growth of S. typhimurium with varying amounts of HAITF. Significant dose-dependent, lag-phase reduction of the growth curves suggests promotion of growth by an iron-chelating mechanism because this is a pattern typical for iron-chelating compounds (12). Fig. 5 B shows the results of similar experiments carried
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Fig. 5. (A) Growth of wild-type S. typhimurium measured by optical density (OD) for various additions of HAITF. Growth recorded every 1–2 h and 1.0 µl (●), 10 µl (△), or 20 µl (×) HAITF was added. (B) Growth of E. coli and K. pneumoniae measured by optical density (OD). Growth was recorded every 1–2 h and 0.1 µl (●), 10 µl (△), or 20 µl (×) HAITF was added. (○) no addition.

out with E. coli and K. pneumoniae. Again, lag-phase reductions are demonstrated and are proportional to the amount of HAITF added to the culture medium.

To further test the hypothesis that growth stimulation of bacteria by HAITF is related to iron chelation, an experiment with a competing iron-chelating compound was performed. Fig. 6 shows the growth curves of TA 2701 in low-iron medium in the presence of HAITF together with varying concentrations of desferrioxamine. Increasing the concentration of desferrioxamine prevents the reduction of the lag phase produced by HAITF. Presumably, desferrioxamine is competing for the iron in the media, making it unavailable to both HAITF and the mutant salmonella.

Effect of HAITF on Bacterial Infections In Vivo. The effect of HAITF on Salmonella infection in mice is seen in Fig. 7 A and B. HAITF potentiates wild-type S. typhimurium infection as seen in Fig. 7 A. No deaths were seen in the noninfected control groups that received injections of HAITF or saline. The survival of the infected group that received HAITF was significantly less than the infected group that received normal saline.

To illustrate competition of this potentiation by another iron-binding compound, the mutant salmonella TA 2701 was used as above as the infectious agent. Fig. 7 B
Fig. 6. Growth curves of *S. typhimurium* TA 2701 in low-iron medium in the presence of HAITF together with varying concentrations of desferrioxamine. (X) 20 µl HAITF; (▲) 20 µl HAITF + 1 µM desferrioxamine; (△) 20 µl HAITF + 10 µM desferrioxamine; (○) no addition; (●) 10 µM desferrioxamine.

Fig. 7. (A) Probability of survival vs. time of mice infected with wild-type *S. typhimurium* and treated with injections of HAITF (○) or saline (●). HAITF injections significantly reduce survival when compared with mice that received saline injections; *P* < 0.001 at 1.9 d. (B) Probability of survival vs. time of mice infected with mutant *S. typhimurium* TA 2701 and treated with injections of HAITF alone (○), HAITF + desferrioxamine (DES) (▲), DES alone (△), or saline (●). Desferrioxamine produces a protective effect in mice also given HAITF when compared with HAITF alone; 0.05 < *P* < 0.10 at 4.25 d. Desferrioxamine treatment alone also produces a protective effect when compared with HAITF alone (*P* < 0.01 at 4.25 d), or with saline injections (*P* < 0.01 at 6.46 d).
shows the results of four groups of mice infected with TA 2701. Each group received HAITF alone (group I), HAITF plus desferrioxamine (25 mg/kg) (group II), desferrioxamine alone (25 mg/kg) (group III), or normal saline (group IV). Group I exhibited reduced survival compared with group IV as in the wild-type infection. Furthermore, groups II and III, which received HAITF and desferrioxamine or desferrioxamine alone, were protected. This suggests a competitive effect between HAITF and desferrioxamine in vivo.

**Discussion**

These experiments indicate the presence of a low molecular weight iron-binding compound in mammalian tissue that is capable of stimulating the growth of enteric bacteria in low-iron medium. The fact that the compound is dialyzable places the molecular weight of the compound at <10,000. Furthermore, results of exclusion from gel filtration columns suggest that the factor has a molecular weight between 1,500 and 5,000. As isolated, the material is associated with iron that can be removed with Chelex-100 resin without loss of bioactivity.

Experiments employing radiolabeled iron indicate that HAITF will bind iron and that this iron migrates with the bioactivity in both a paper chromatographic system and on gel filtration columns. It is interesting that in the paper chromatographic system the iron-staining spot migrates somewhat behind the radioactivity whereas both peaks are associated with bioactivity. This suggests that the iron-bound form of the molecule is less polar than the apo-form of HAITF.

Our results also show a lag-phase reduction of the growth curve of wild-type *S. typhimurium*, *E. coli*, and *K. pneumoniae* when grown in the presence of HAITF. This pattern has frequently been reported with iron-binding compounds of microbial origin, both catecholates and hydroxamates (13). We have also shown an inhibition of growth stimulation by desferrioxamine in an organism unable to utilize the iron chelate of desferrioxamine. Additional evidence that iron chelation plays a role in the interaction of HAITF and bacteria is supplied by the iron-uptake experiments. Iron uptake by resting wild-type *S. typhimurium* was increased by the addition of HAITF to the medium. Because the organisms were grown in low-iron medium, they were undoubtedly producing enterobactin so that addition of HAITF stimulates iron uptake over and above the inherent potential of the bacteria. Stimulation of iron uptake by HAITF was also inhibited by desferrioxamine in the mutant salmonella unable to utilize desferrioxamine as an iron donor. Again these results indicate HAITF can act as an iron donor to bacteria.

In vitro promotion of bacterial growth by HAITF is mirrored in vivo by the results shown in Fig. 7 A and B. Theoretically, the amounts of HAITF injected were enough to raise the extracellular bioactivity of the host to twice the normal level. The results show that HAITF injections significantly reduce the survival of infected animals. As in the in vitro experiment, the mutant model suggests that increased bacterial growth manifested by reduced host survival is secondary to increased iron uptake by the pathogen. It is possible that injections of semipurified HAITF exert a toxic effect on the animals that lowers their resistance to the pathogen and that the protective effect of desferrioxamine is not associated with competition for iron with HAITF. However, the animals that received HAITF alone that were not infected showed no deaths or signs of toxicity.
HAITF bioactivity was found in all tissues examined that included serum. It appears to be an ubiquitous substance, but the highest levels were found in the liver and spleen. This may be significant in light of the higher level of iron stores in these tissues.

Very little is known about small molecular weight iron-binding molecules in mammalian tissues or their interaction with bacteria. It is known that compounds used to treat iron overload such as desferrioxamine or 2,3-dihydroxybenzoic acid can promote the virulence of microorganisms by the iron-chelating properties of the drug (8). We have described a low molecular weight factor in mammalian tissue with iron-binding properties that is capable of promoting bacterial growth. The physiological function of HAITF activity may be involved in cellular iron transport, a phenomenon that is not well characterized. However, whatever its function, HAITF may inadvertently supply iron to invading pathogens. Further work is needed to define its structure, its role in normal iron metabolism, and its relationship to the process of infection.

Summary

A low molecular weight, iron-binding factor was isolated from horse liver. This host-associated iron transfer factor (HAITF) is capable of binding iron and stimulating bacterial growth by promoting iron uptake into bacteria. Also, when injected into infected animals, HAITF increases the virulence of bacterial infections. HAITF bioactivity is ubiquitous in animal tissues and present in serum. It is proposed that HAITF is a factor that inadvertently plays a role in the host-parasite competition for iron.

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