Specific Inhibition of *Escherichia coli* Ferrienterochelin Uptake by a Normal Human Serum Immunoglobulin

DAVID G. MOORE AND CHARLES F. EARHART*

Department of Microbiology, The University of Texas at Austin, Austin, Texas 78712

Normal human serum contains an enterochelin-specific antibody which presumably acts with transferrin to hinder iron assimilation by enterochelin-producing pathogens. This antibody can be isolated from serum by sodium sulfate fractionation or affinity chromatography by employing an enterochelin-derived ligand (2,3-dihydroxy-*N*-benzoyl-L-serine) attached to aminohexyl Sepharose 4B. In assays of iron uptake by whole cells, the antibody inhibited enterochelin-directed uptake but not that mediated by citrate or ferrichrome. Also, the growth stimulatory effect of enterochelin on an Ent⁻ strain of *Escherichia coli* was blocked by the immunoglobulin. This antibody has a high affinity for enterochelin; various elution procedures employing high salt concentrations and low pH failed to remove it from affinity columns. Elution with 3 M sodium thiocyanate or 13 mM 2,3-dihydroxybenzoic acid proved successful. Two pieces of evidence indicate the enterochelin-specific antibody is primarily of the immunoglobulin A (IgA) isotype. It could be removed from serum with goat antihuman IgA and was present only in sodium sulfate fractions of serum known to contain IgA.

Normal human serum is bactericidal for a variety of microorganisms because of the lytic action of the complement pathway. Moreover, many bacteria have difficulty growing in serum even when complement has been inactivated. This latter phenomenon is thought to be the result of iron deprivation; the iron in serum is essentially all bound to the iron-binding protein transferrin (15). To obtain the essential metal, pathogens must synthesize siderophores (specialized iron-solubilizing agents) to remove iron from transferrin. The siderophore produced by *Escherichia coli* and *Salmonella typhimurium* is enterochelin (enterobactin) (11, 12), a cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine (DBS). Enterochelin will stimulate growth of *S. typhimurium* in complement-inactivated human serum (16) and enhance the virulence of the organism in mice (18).

Recent work (10) demonstrated that human serum contains a naturally occurring antibody specific for enterochelin that presumably acts in concert with transferrin to limit the growth of enterochelin-producing pathogens. This immunoglobulin can be removed from heat-inactivated serum by incubation with ultraviolet light-killed cells that had the potential to produce enterochelin or by inert affinity matrices to which enterochelin-derived ligands had been attached. Bacteriostasis is partially restored to a defined medium supplemented with transferrin by addition of the immunoglobulin fraction isolated from normal human serum.

We report here that the bacteriostatic antibody isolated by affinity chromatography is of the immunoglobulin A (IgA) isotype and inhibits ferrienterochelin uptake but does not affect iron assimilation mediated by ferrichrome or citrate.

MATERIALS AND METHODS

Bacteria. The organisms used were *E. coli* RW193 *entA* (unable to synthesize enterochelin [Ent⁻]) (6), *E. coli* AN102 *fep* (unable to transport ferrienterochelin) (2), and *S. typhimurium* 96-1 (Ent⁻) (10, 18).

Chemicals. Aminohexyl Sepharose 4B (AHS4B) was purchased from Pharmacia Fine Chemicals, Inc., and 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide was obtained from Bio-Rad Laboratories. Crystalline human transferrin was purchased from Sigma Chemical Co., and goat anti-human IgA antiserum was purchased from Miles Laboratories. Ferrichrome was obtained from Porphyrin Products.

Growth of bacteria. Cultures were maintained at 4°C on brain heart infusion agar (Difco Laboratories). Inocula for growth studies were prepared as previously described (10). RW193 cells for ⁵⁵Fe uptake assays were prepared as described elsewhere (13) except that appropriately supplemented M9 medium (9) which had been iron depleted was used for overnight cultures and subcultures. M9 medium was rendered iron free by hydroxyquinoline-chloroform extraction (14). RW193 cells for the assay of citrate-mediated ⁵⁵Fe uptake were grown in supplemented M9 medium containing 1 mM sodium citrate.

Tissue culture medium 199 (Difco) was prepared to mimic immunoglobulin-free serum as before (10). A sterile solution of apotransferrin in 10 mM sodium bicarbonate was prepared and added at physiological

concentration (30 µM) to medium 199 to provide the nonspecific, bacteriostatic component of serum.

Serum. Serum for growth studies and immunoglobulin isolation was isolated from healthy human volunteers and complement inactivated as previously described (10). Glassware used in experiments involving serum, enterochelin, or iron-depleted medium was iron free (18).

Iron uptake assay. The uptake of ^{55}Fe by RW193 was measured by methods previously described (8).

Spectrophotometric assays. Iron saturation levels of serum transferrin were determined in all sera by the methods of Williams and Conrad (17) employing the chromagens 2,4,6-tripyridyl-S-triazine and 2,2',2"-tripyridyl. Protein concentration was determined by the method of Lowry (7). Enterochelin and DBS concentrations were determined by the methods of Arnow (1) or from the molar extinction coefficient at 315 nm (11).

Enterochelin and DBS. Enterochelin and DBS were isolated from the supernatant fluid of a late-log-phase culture of $E.$ $coli$ AN102 by ethyl acetate extraction (10).

Isolation of immunoglobulin. Isolation of immunoglobulin fractions from serum was by the sodium sulfate precipitation method (5).

Preparation of coupled ligands. DBS or purified goat anti-human IgA antibody was coupled to AHS4B by the carbodiimide coupling procedure recommended by Pharmacia.

Adsorption of serum and affinity chromatography. To adsorb serum, heat-inactivated human serum (5 ml) was incubated with 1 ml of AHS4B-anti-IgA for 1 h at 37°C. The ligand was collected by centrifugation, and the serum was then filter sterilized.

Affinity chromatography was used to isolate enterochelin-specific antibody; 10-ml portions of human serum were filtered through an AHS4B-DBS column (0.9 by 15 cm). After each portion was filtered, the column was washed with phosphate-buffered saline, and the antibody was eluted by washing the column with either 13 mM dihydroxybenzoic acid (DBA) or 3 M sodium thiocyanate dissolved in 0.15 M NaCl, 0.1 M K_2HPO_4, and 0.005 M KH_2PO_4 (3). The DBA or thiocyanate eluates from 100 ml of serum were collected, concentrated by ultrafiltration (Amicon PM-10 membrane), and dialyzed successively against phosphate-buffered saline, iron-depleted M9 medium supplemented with 10 mM ethylenediaminetetraacetic acid (EDTA), and iron-depleted M9 medium. Eluates were sterilized by passage through Whatman GF/A filters.

RESULTS

The effect of enterochelin-specific antibody on bacterial iron assimilation was studied by using a radioactive iron uptake assay directed by enterochelin. The cells used (strain RW193) are unable to synthesize enterochelin and the presence of nitrilotriacetic acid prevented low affinity iron uptake; iron uptake was thus dependent on the exogenously added siderophore. Iron-free enterochelin and various immunoglobulin preparations were added, and the time

course of ^{55}Fe uptake was determined. As shown in Fig. 1A, 0.25 µM enterochelin resulted in the uptake of approximately 16 ng of ^{55}Fe per mg (dry weight) of cells, whereas no cellular iron accumulation occurred in the absence of enterochelin. Addition of the immunoglobulin fraction isolated by sodium sulfate precipitation of serum to the iron uptake assay to a final concentration of 7 mg/ml reduced uptake to a maximum of 2 ng of ^{55}Fe per mg (dry weight) of cells in the presence of enterochelin.

The iron uptake assay was then modified to resemble the conditions of serum with respect to iron availability by initially chelating the ^{55}Fe to the serum iron-binding protein transferrin (30 µM) rather than to nitrilotriacetic acid. As shown in Fig. 1B, approximately 15 ng of ^{55}Fe per mg (dry weight) of cells was taken up in the presence of enterochelin, compared with no iron uptake in its absence. Addition of human serum immunoglobulin (7 mg/ml) inhibited enterochelin-directed ^{55}Fe uptake by about 90%.

The enterochelin-specific antibody was isolated from the total immunoglobulin fraction by affinity chromatography. Serum was filtered through an AHS4B-DBS column and washed with phosphate-buffered saline. Initially, a variety of elution procedures employing combinations of high salt concentration and low pH failed to remove the antibody from the column as judged by (i) continued saturation of the column with antibody and (ii) failure of the eluate to inhibit ^{55}Fe uptake. Elution of immunoglobulin with either the chaotropic agent so-
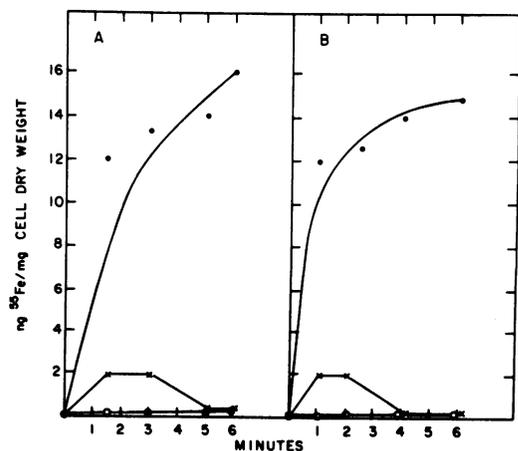

FIG. 1. *Effect of human immunoglobulin on enterochelin-mediated ^{55}Fe uptake by E. coli RW193. The iron was initially associated with nitrilotriacetic acid (A) or transferrin (B). The assay mixture was supplemented as follows:* ●, *0.25 µM enterochelin;* ×, *0.25 µM enterochelin and immunoglobulin (7.1 mg/ml);* ○, *no supplements.*

dium thiocyanate or DBA, which has structural similarities to DBS and enterochelin, proved successful. The dialyzed thiocyanate eluate containing the antibody was added to the iron uptake assay, and its effects are shown in Fig. 2A. The iron uptake permitted by 2.5 μM enterochelin (50 ng of ^{55}Fe per mg [dry weight] of cells) could be inhibited by the thiocyanate eluate (1.71 mg/ml). The dialyzed DBA eluate had a similar effect on iron uptake as shown in Fig. 2B. These data indicate that it is possible to remove the antibody from the column through antigenic competition with DBA as well as by physical dissolution of the antigen-antibody complexes with sodium thiocyanate. Furthermore, antibody isolated from an AHS4B-DBS affinity column does inhibit enterochelin-directed ^{55}Fe uptake.

E. coli has two other high-affinity iron transport systems in addition to that which uses enterochelin; one employs the heterologous siderophore ferrichrome and the other is mediated by citrate. The antibody isolated by thiocyanate elution was tested for its effect on ^{55}Fe uptake directed by these auxiliary systems. The addition of 15 mg of the antibody preparation per ml had no effect on ^{55}Fe uptake directed by ferrichrome (0.25 μM) (Fig. 3A) or by citrate (10 mM) (Fig. 3B). These data provide further evidence that the antibody specifically inhibits enterochelin-directed iron uptake.

The ability of the thiocyanate eluate from AHS4B-DBS affinity columns to specifically inhibit enterochelin-directed iron uptake sug-
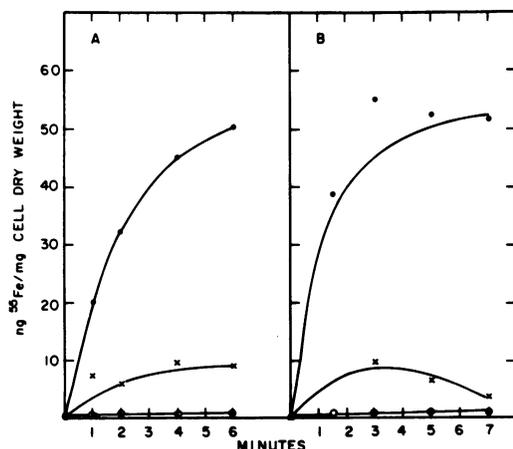

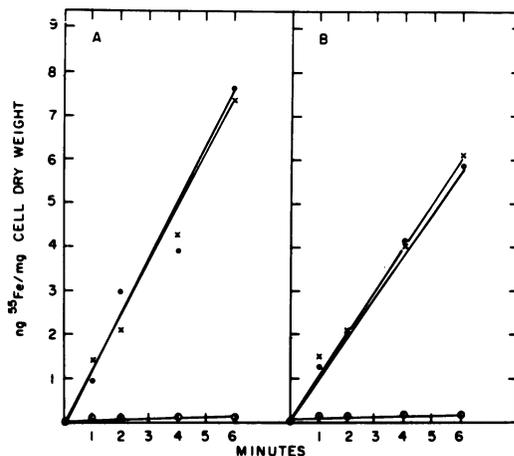FIG. 3. *Effect of antibody eluted from AHS4B-DBS columns by thiocyanate on E. coli RW193 ^{55}Fe-ferrichrome (A) and ^{55}Fe-citrate (B) uptake. The uptake mixtures were supplemented as follows:* ●, *ferrichrome (A), citrate (B);* ×, *siderophore and immunoglobulin (15 mg/ml);* ○, *no supplement.*

gested that the antibody could inhibit the growth stimulatory effect of enterochelin in an iron-restricted environment. The immunoglobulin was added in a physiological concentration to medium 199 supplemented with 30 μM transferrin and 0.05 μM enterochelin. The reconstituted medium (medium 199, apotransferrin, immunoglobulin, enterochelin) was 250-fold more bacteriostatic for RW193 than medium without added immunoglobulin (medium 199, apotransferrin, enterochelin) (Fig. 4). Strain RW193 failed to grow in antibody-free medium without enterochelin (medium 199, apotransferrin).

The following data suggest strongly that the enterochelin-specific antibody is of the IgA isotype. The principal isotypes (IgG, IgA, and IgM) of serum antibody can be isolated by successive sodium sulfate fractionations of serum; the 18% cut contains the total immunoglobulin fraction, the 16% cut contains primarily IgG and IgA, and the 12% cut contains almost solely IgG. These fractions were added separately to the enterochelin-directed iron uptake assay, and the time course of iron uptake was followed. Iron uptake (Fig. 5) was inhibited most by the total antibody fraction (18%) and the 16% cut (IgA and IgG); the 12% cut was least inhibitory. These data suggested that the antibody responsible for iron uptake inhibition was not IgG but, more likely, IgA. This idea was substantiated by monitoring the growth of S. typhimurium 96-1 in human serum that had been adsorbed with an affinity matrix composed of goat anti-human IgA coupled to AHS4B. The results shown in Fig. 6
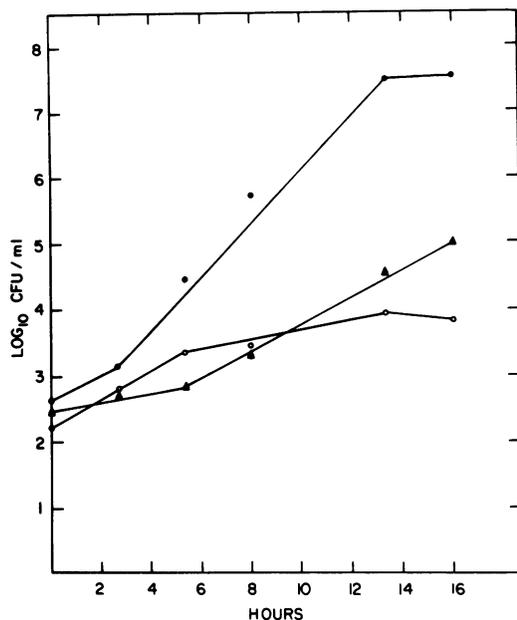

FIG. 2. *Effect of antibody eluted from AHS4B-DBS affinity columns with 3 M sodium thiocyanate (A) or 13 mM DBA (B) on ^{55}Fe uptake by E. coli RW193. The assay mixture was supplemented as follows:* ●, *2.5 μM enterochelin;* ×, *2.5 μM enterochelin and immunoglobulin (1.71 mg/ml);* ○, *no supplement.*

FIG. 4. *Antibody (thiocyanate eluate of AHS4B-DBS column) inhibition of the stimulatory effect of enterochelin (0.05 µM) on E. coli RW193 growth in defined medium. Symbols:* ●, *medium 199, apotransferrin, and enterochelin;* ▲, *medium 199, apotransferrin, enterochelin, and immunoglobulin (7 mg/ml):* ○, *medium 199 and apotransferrin.*

show that removal of IgA from serum by affinity chromatography permits growth.

DISCUSSION

The current results further characterize the enterochelin-specific antibody present in normal human serum. This work was facilitated by the observation that the antibody prevents enterochelin-mediated iron uptake. This new assay for the antibody was more rapid and less cumbersome than the previous method, which depended on the fact that heat-inactivated serum containing the immunoglobulin is bacteriostatic for *S. typhimurium* 96-1 and Fisher. That the antibody blocked enterochelin-mediated uptake but not that provided by ferrichrome or citrate not only verified the specificity of the antibody but also eliminated the possibility that inhibition of ferrienterochelin uptake by IgA fractions was a nonspecific artifact. In addition, the uptake experiments provided information regarding the mechanism by which the enterochelin-specific antibody brings about bacteriostasis. The antibody blocked iron uptake even though enterochelin was added exogenously to the assay mixture; thus the antibody did not act at the level of enterochelin synthesis or excretion.

The immunoglobulin is primarily of the IgA

isotype; (i) it was removed from serum by affinity matrices to which goat antihuman IgA had been attached, and (ii) only those sodium sulfate fractions of human serum containing IgA were strongly inhibitory to enterochelin-directed ^{55}Fe uptake. The antibody bound enterochelin strongly, as demonstrated by the failure of normal elution conditions such as high salt concentrations and low pH to remove it from affinity matrices, and it inhibited enterochelin-dependent growth (Fig. 4).

We have no direct evidence regarding the origin of these antibodies. However, lymphoid cells (predominantly with IgA), as isolated cells, small cell clusters, or lymphoepithelial structures (Peyer's patches), are present in the intestinal walls. There they presumably have the opportunity to be antigenically stimulated by resident *E. coli* cells bearing enterochelin. The nature of the actual immunogenic complex remains unknown. However, the ability of ultraviolet light-killed Ent$^+$ whole cells to remove the antibody from serum (10) suggests that enterochelin is firmly bound to a cell envelope component. The complex present on ultraviolet light-killed Ent$^+$ cells and the immunogenic complex are presumably related. The envelope
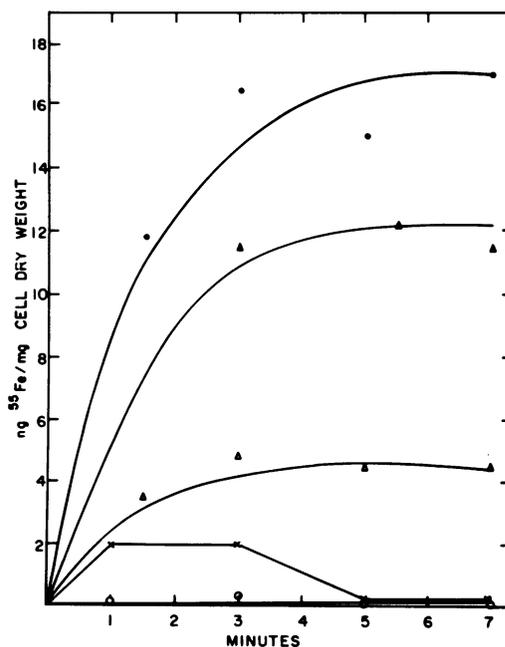

FIG. 5. *Effect of sodium sulfate immunoglobulin fractions (7 mg/ml) from human serum on 0.25 µM enterochelin-directed* ^{55}*Fe uptake by E. coli RW193. Symbols:* ●, *no antibody;* ▲, *12% cut (IgG);* △, *16% cut (IgG and IgA);* ×, *18% cut (IgG, IgM, and IgA);* ○, *no enterochelin or immunoglobulin added.*
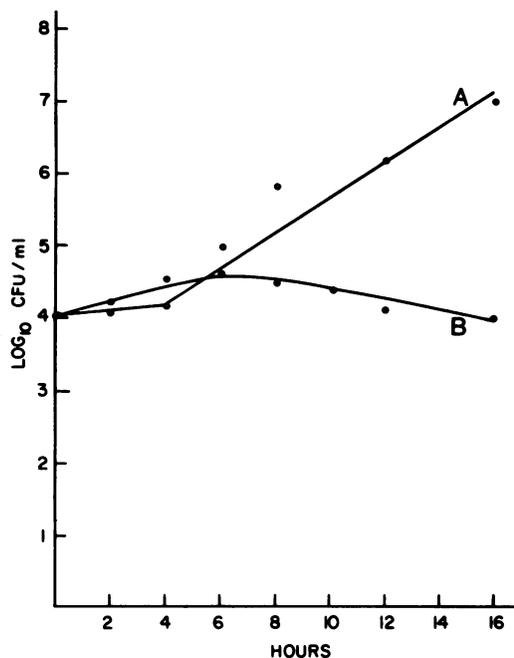
FIG. 6. *Growth of S. typhimurium 96-1 in serum adsorbed with goat anti-human IgA attached to AHS4B. Symbols: O, adsorbed, line A; ●, unadsorbed, line B.*

component is not lipopolysaccharide; it was previously demonstrated (10) that the component is heat labile, and Ent⁺ *E. coli* and *S. typhimurium* strains with severe lipopolysaccharide defects still adsorb out the antibody (Moore, unpublished data).

Fitzgerald and Rogers (4) have studied IgG in serotype-specific horse antiserum and secretory IgA in human milk that act with transferrin to exert a bacteriostatic effect. These antibodies recognize colitose, the terminal sugar of the lipopolysaccharide of *E. coli* 0111, which was the test organism. Fitzgerald and Rogers have suggested that these antibodies interfere with enterochelin synthesis or secretion. This system may represent an alternative immune mechanism for depriving cells of iron; it differs in all particulars from that which we have described.

ACKNOWLEDGMENTS

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