Effect of Immunoglobulin G from Cows Immunized with Ferric Citrate Receptor (FecA) on Iron Uptake by *Escherichia coli*¹

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ABSTRACT

The effects of immunoglobulin (Ig) G from cows immunized with the ferric citrate receptor (FecA) on iron uptake by *Escherichia coli* were investigated. Receptor FecA was purified from *E. coli* UT5600/pSV66. Cows were immunized with 400 μ g purified FecA three times at 21 d intervals during late lactation and the nonlactating period. Immunoglobulin G was purified by protein G affinity chromatography from colostrum whey from cows immunized with FecA and from unimmunized control cows. The purified IgG from FecA immunized cows had higher IgG titers against FecA compared with control IgG. Fifteen *E. coli* isolated from intramammary infections and *E. coli* UT5600/pSV66 were grown in an iron-depleted medium containing 1 mM citrate to induce FecA. The bacterial cells were mixed with 0, 2, and 4 mg/ml purified IgG, and ⁵⁵Fe was added to the assay. After 5, 10, and 15 min incubations at 37°C, samples were passed through 0.45- μ m pore size filters. Filters were washed with saline three times, and the radioactivity of ⁵⁵Fe taken up by the bacterial cells on the filters was measured by a liquid scintillation counter. The measurements were expressed as numbers of ⁵⁵Fe atoms per colony-forming unit and transformed to log₁₀. The assay was repeated three times for each isolate in a partially balanced incomplete block design. The presence of IgG decreased ⁵⁵Fe uptake by *E. coli* mastitis isolates and *E. coli* UT5600/pSV66. Anti-FecA IgG reduced ⁵⁵Fe uptake by *E. coli* greater than IgG from unimmunized cows. (**Key words:** bacterial iron uptake, FecA, immunoglobulin, *Escherichia coli*)

Abbreviation key: TSB = trypticase soy broth, IROMP = iron regulated outer membrane protein.

INTRODUCTION

Iron is essential to maintain normal cellular metabolism for almost all bacteria. Under iron-restricted conditions, *Escherichia coli* utilize multiple iron transport systems and express iron regulated outer membrane proteins (IROMP) that bind to and internalize ferric compounds to acquire iron (van der Helm, 1998). Ferric citrate receptor, FecA, is an IROMP of *E. coli* that facilitates uptake of ferric citrate (Wagegg and Braun, 1981). FecA can be induced with more than 0.1 mM citrate under iron-restricted conditions (Frost and Rosenberg, 1973). Bovine milk contains approximately 7 mM citrate (Faulkner and Peaker, 1982). The concentration of citrate in milk is sufficient to form ferric citrate, a specific ligand for FecA (Hussein et al., 1981), and to induce the ferric citrate transport system in bacteria (Frost and Rosenberg, 1973). *Escherichia coli* isolates from cases of naturally occurring mastitis expressed FecA when bacteria were cultured in concentrations of citrate comparable to that in the lactating bovine mammary gland (Lin et al., 1999).

Iron regulated outer membrane proteins are vaccine candidates because they are surface exposed, immunogenic, and may induce protective immunity (Byers and Arceneaux, 1998). The immunization of cows with FecA increased antibody titers against FecA in serum and whey (Takemura et al., 2002). Although the effect of FecA immunization in lactating cows was minimal following *E. coli* intramammary challenge, increased antibody titers against FecA were associated with decreased peak bacterial number in milk from challenged quarters (Takemura et al., 2002). The objective of this study was to investigate effects of purified IgG from cows immunized with FecA on the in vitro iron acquisition of *E. coli*.

MATERIALS AND METHODS

Immunization

Immunizations were as described previously (Takemura et al., 2002). Seven cows were immunized with 400 μ g of purified FecA three times: 1) subcutaneous injection 14 d prior to drying off, 2) intramammary infu-

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sion 7 d after drying off, and 3) subcutaneous injection 28 d after drying off. Seven cows served as unimmunized controls. The FecA vaccine was derived from *E. coli* UT5600/pSV66 (Lin et al., 1999) and administered as described by Takemura et al. (2002).

Immunoglobulin Purification

Colostrum was collected from each cow within 12 hr after parturition. Immunoglobulin G was purified from pooled colostrum whey of FecA immunized cows and unimmunized control cows by ammonium sulfate precipitation followed by Protein G affinity chromatography. The sepharose gel, Protein G Sepharose 4 Fast Flow (Pharmacia Biotech AB, Uppsala, Sweden), was packed into 1.5 × 5 cm column and equilibrated with the binding buffer (20 mM of sodium phosphate buffer, pH 7.0). Protein applied to the bed volume was 20 mg/ml, and the flow rate was 0.8 ml/min. After the binding, the column was washed with 10 bed volumes of the binding buffer. The bovine IgG bound to the sepharose gel was eluted with 10 bed volumes of 0.1 M glycine-HCl buffer (pH 2.7). To neutralize the eluted fractions, 100 μ l/ml of 1M Tris-HCl (pH 9.0) was added in the tubes prior to the elution. The fractions high in protein were pooled and dialyzed against PBS. The concentration of purified IgG was measured by bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL). The purity of IgG was determined by SDS-PAGE with the control of commercially purified bovine IgG (Sigma Chemical Co., St. Louis, MO). Titers against FecA for purified IgG (4 mg/ml) were determined by ELISA as described previously (Takemura et al., 2002). The purified IgG was stored at -20°C prior to use. All glassware was washed in 0.1 N concentrated nitric acid for 4 h and rinsed three times in distilled, deionized water.

Bacterial Strains and Experimental Design

Fifteen isolates of *E. coli* from cases of naturally occurring bovine intramammary infections and *E. coli* strain UT5600/pSV66 (*leu*⁻, *proC*⁻, *trpE*⁻, *entA*⁻, *rpsI*⁻, Δ (*ompT-fepA*) / *Cmr fecIRA*) were tested (Lin et al., 1999). Bacteria were stored in trypticase soy broth (TSB) containing 20% glycerin at -70°C. The iron transport assay was repeated three times for each *E. coli* mastitis isolate and for *E. coli* UT5600/pSV66 using a partially balanced incomplete block design.

Expression and Detection of FecA

Prior to the assay, the expression of FecA by *E. coli* mastitis isolates and *E. coli* UT5600/pSV66 was determined. Bacteria were cultured in TSB at 37°C for 18 h.

Bacteria were washed with PBS and cultured in TSB containing 200 μ M α - α' -dipyridyl and 1 mM citrate at 37°C for 3 h to induce FecA. The outer membrane proteins were isolated (Todhunter et al., 1991) and separated by SDS-PAGE (12.5% polyacrylamide) by the method of Laemmli (1970). Immunoblots were performed to detect FecA as described by Lin et al. (1999) with the following exceptions. The primary antibody was 0.4 mg/ml bovine IgG and the secondary antibody was horseradish peroxidase-conjugated goat anti-bovine IgG (1:400, vol/vol. Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Iron Transport Assay

Escherichia coli were cultured as described above. Bacteria were then washed with PBS and suspended in H medium (Hussein et al., 1981) to an optimal density of 0.5 at a wavelength of 578 nm. A portion of the bacterial suspension was serially diluted in PBS and cultured on MacConkey agar plates to determine colony-forming units. Nitrilotriacetate was added to the assay at a final concentration of 0.1 mM to suppress nonspecific iron uptake (van Hove et al., 1990). Bacteria were mixed with anti-FecA IgG or control IgG at the concentrations of either 0, 2, or 4 mg/ml in H medium for 30 min on ice. The ⁵⁵Fe cocktail was added to the assay at a final concentration of 1 μ M ⁵⁵Fe, 10 μ M FeCl₃, and 100 μ M sodium citrate, and incubated at 37°C. Samples of 400 μ l containing 5 × 10⁷ cfu of *E. coli* were taken from the assay after 5, 10, and 15 min of incubation. The cells were harvested on cellulose nitrate filters (pore size 0.45 μ m) and washed 3 times with saline. Filters were placed in a liquid scintillation counter cocktail (EcoLite (+), ICN Pharmaceuticals, Inc., Costa Mesa, CA) and the radioactivity was measured by liquid scintillation counting (6892 Liquid Scintillation Systems, Tracor Analytic, Elk Grove Village, IL). The measurements were expressed as numbers of ⁵⁵Fe atoms taken up per colony-forming unit, and transformed to log¹⁰ for statistical analysis.

Statistical Analysis

Treatment differences for log₁₀ ⁵⁵Fe atoms/cfu were tested by least squares ANOVA (SAS Inst. Inc., 1999). The variable was tested for treatment effects partitioned into the orthogonal contrasts: 1) bacterial ⁵⁵Fe uptake without IgG versus bacterial ⁵⁵Fe uptake with IgG, and 2) bacterial ⁵⁵Fe uptake with anti-FecA IgG versus bacterial ⁵⁵Fe uptake with control IgG.

RESULTS

Immunoglobulin G Titers Against FecA

Immunoglobulin G titers against FecA were 1:5120 for anti-FecA IgG and 1:64 for control IgG. The concentration of IgG used for the ELISA was 4 mg/ml in PBS.

Expression and Detection of FecA

The SDS-PAGE gel of outer membrane proteins isolated from *E. coli* demonstrated the expression of IROMP with molecular weight of approximately 80 to 81 kDa when the bacteria were grown in TSB containing 200 μ M α - α' -dipyridyl and 1 mM citrate for 3 h (Figure 1a). Anti-FecA IgG (0.4 mg/ml) detected the protein with a molecular weight of approximately 80.5 kDa (Figure 1b); however, control IgG (0.4 mg/ml) did not recognize IROMP (data not shown).

Iron Uptake by *E. coli* Mastitis Isolates

The uptake of ^{55}Fe by *E. coli* mastitis isolates increased from 5 to 15 min incubation for all treatments (Figure 2). The presence of IgG reduced ^{55}Fe uptake of *E. coli* mastitis isolates compared with ^{55}Fe uptake in the H medium after 5, 10, and 15 min of incubation ($P < 0.001$). Anti-FecA IgG reduced ^{55}Fe uptake by *E. coli* mastitis isolates compared with control IgG at each sampling time ($P < 0.001$). The uptake of ^{55}Fe by *E. coli* mastitis isolates did not differ between 2mg/ml and 4 mg/ml of

Figure 1. Outer membrane profiles of *Escherichia coli* separated by SDS-PAGE and stained with Coomassie blue (A). Protein per lane was 10 μ g. Bacteria were cultured in trypticase soy broth (TSB) or TSB containing 200 μ M α - α' -dipyridyl and 1 mM citrate (TSB-cit). Lane contents were 1) molecular weight standards ($\times 10^3$ Dalton); 2) *E. coli* 92, TSB; 3) *E. coli* 92, TSB-cit; 4) *E. coli* 471, TSB; 5) *E. coli* 471, TSB-cit; 6) *E. coli* 727, TSB; 7) *E. coli* 727, TSB-cit; 8) *E. coli* UT5600/pSV66, TSB; 9) *E. coli* UT5600/pSV66, TSB-cit. Approximate position of iron-regulated outer membrane proteins (IROMP) is indicated. Immunoblots of separated outer membrane proteins of *E. coli* reacted with 0.4 mg/ml IgG from cows immunized with FecA (B). Protein per lane was 10 μ g. Lane contents were 1) *E. coli* 92, TSB; 2) *E. coli* 471, TSB; 3) *E. coli* 727, TSB; 4) *E. coli* UT5600/pSV66, TSB; 5) *E. coli* 92, TSB-cit; 6) *E. coli* 471, TSB-cit; 7) *E. coli* 727, TSB-cit; 8) *E. coli* UT5600/pSV66, TSB-cit. Approximate position of FecA is indicated.

IgG for either anti-FecA or control IgG ($P > 0.05$). Uptake of ^{55}Fe differed among *E. coli* mastitis isolates at each sampling time ($P < 0.05$).

Iron Uptake by *E. coli* UT5600/pSV66

Uptake of ^{55}Fe by *E. coli* UT5600/pSV66 increased from 5 to 15 min incubation for all treatments (Figure 3). The presence of IgG reduced ^{55}Fe uptake by *E. coli* UT5600/pSV66 compared with ^{55}Fe uptake in the H medium after 5, 10, and 15 min of incubation ($P < 0.001$). At each sampling time, uptake of ^{55}Fe by *E. coli* UT5600/pSV66 was further reduced in the presence of anti-FecA IgG compared with control IgG ($P < 0.001$). Uptake of ^{55}Fe by *E. coli* UT5600/pSV66 did not differ between 2 mg/ml and 4 mg/ml of IgG for both IgG sources ($P > 0.05$).

DISCUSSION

Escherichia coli expressed FecA, thus suggesting that the isolates utilized the ferric citrate transport system to acquire iron. Anti-FecA IgG reduced uptake of ^{55}Fe by *E. coli* compared with control IgG. Uptake of ^{55}Fe by mastitis isolates in the presence of IgG from unimmunized cows was comparable to ^{55}Fe uptake by mastitis isolates in a medium devoid of antibodies. These responses support the hypothesis that FecA is antigenic

Figure 2. Mean number of ^{55}Fe atoms ($\log_{10}$ number ^{55}Fe atoms per colony-forming unit) taken up by *Escherichia coli* mastitis isolates ($n = 15$) in the presence of 2mg/ml control IgG (○), 4mg/ml control IgG (▼), 2 mg/ml anti-FecA IgG (▽), 4 mg/ml anti-FecA (■), and devoid IgG (●). Dispersion bars represent standard error.

Figure 3. Mean number of ^{55}Fe atoms ($\log_{10}$ number ^{55}Fe atoms per colony-forming unit) taken up by *Escherichia coli* UT5600/pSV66 in the presence of 2 mg/ml control IgG (○), 4 mg/ml control IgG (▼), 2 mg/ml anti-FecA IgG (▽), 4 mg/ml anti-FecA (■), and devoid IgG (●). Dispersion bars represent standard error.

and that the IgG from immunized cows may disrupt the uptake of ferric citrate by *E. coli*.

Despite the reduction of ^{55}Fe uptake, anti-FecA IgG did not prohibit the uptake of ^{55}Fe . Under iron-restricted conditions, *E. coli* utilizes multiple iron transport systems (Braun et al., 1998). *Escherichia coli* mastitis isolates commonly utilize FepA- and enterobactin-based iron transport systems under iron-restricted conditions (Lin et al., 1998). FepA expressed by *E. coli* isolates from cases of naturally occurring mastitis was antigenically distinct from FecA (Lin et al., 1999). However, when 1mM citrate was added to the iron-depleted culture medium, *E. coli* K-12 already expressing FepA also induced FecA, resulting in *E. coli* K-12 expressing both FepA and FecA simultaneously (Wagegg and Braun, 1981). The *E. coli* mastitis isolates grown in an iron-depleted medium containing citrate might have also expressed FepA and secreted enterobactin to uptake iron in the assay. Therefore, *E. coli* mastitis isolates could have transported ^{55}Fe as ^{55}Fe -enterobactin complexes through FepA.

Escherichia coli UT5600/pSV66 lacks the gene *fepA*, therefore does not utilize FepA and enterobactin based iron transport system. When iron exists in a bacterial environment in the order of 10^{-6} M or greater, *E. coli* utilize a low affinity iron transport system based on a passive transport (Guerinot, 1994). To suppress the nonspecific iron transport system, 0.1 mM nitrilotriace-

tate was added to the iron transport assay (Frost and Rosenberg, 1973). Suppression of the nonspecific iron transport system of *E. coli* should have resulted in the ferric citrate transport system utilizing FecA as the sole iron transport system for *E. coli* UT5600/pSV66 in the assay. Anti-FecA IgG reduced ^{55}Fe uptake by *E. coli* UT5600/pSV66 and the reduction was greater than that observed with the mastitis isolates. However, *E. coli* UT5600/pSV66 did acquire ^{55}Fe in the presence of anti-FecA IgG. The surface topology of FecA was suggested to be similar to FepA, and FepA possesses surface loops that have specific binding sites to its ligand, ferric enterobactin (Braun et al., 1998). Among the panel of monoclonal antibodies raised against FepA, only 4 monoclonal antibodies that recognized the cell surface exposed region specifically blocked the interaction of FepA and ferric enterobactin (Murphy et al., 1990). In the present study, cows were immunized with the whole FecA protein. Although the titers against whole FecA protein were approximately 1000 times greater for anti-FecA IgG than control IgG, the actual amount of IgG directed against ligand binding epitopes of FecA that transport iron might have been insufficient to prohibit iron uptake of *E. coli* UT5600/pSV66 and the mastitis isolates.

Immunoglobulin G from FecA immunized cows detected outer membrane proteins with molecular weights of approximately 36 to 38 kDa by immunoblotting. Because the FecA protein used to immunize cows were purified from *E. coli* UT5600/pSV66, the purified protein did not contain FepA. However, the purified FecA may have contained other outer membrane proteins, such as porins. The two porins commonly expressed in *E. coli* are OmpF and OmpC and they are homotrimers of subunits with molecular weights of approximately 36 to 38 kDa (Nikaido, 1994). These porins form water filled channels that allow small substances of molecular weights roughly up to 600 Da to diffuse through spontaneously (Nikaido, 1994). *Escherichia coli* strains that lacked FecA expression still grew under high concentration of citrate because ferric citrate diffused through porin channels in sufficient quantity to support growth (Harle et al., 1995). However, the effect of classical porin system for ^{55}Fe diffusion should have been minimal as the concentration of citrate used in this study was the minimum required to maintain the ferric citrate transport system.

CONCLUSIONS

Immunoglobulin G from cows immunized with FecA decreased uptake of ^{55}Fe by *E. coli* compared with control IgG. These data suggest a specific immune response to FecA reduced iron uptake via the ferric citrate system.

However, anti-FecA IgG did not prohibit the uptake of ^{55}Fe . *Escherichia coli* may utilize alternative high affinity or low affinity iron transport systems under iron-restricted conditions optimal for expression of FecA.

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REFERENCES

- Braun, V., K. Hantke, and W. Koster. 1998. Bacterial iron transport: mechanisms, genetics, and regulation. *Met. Ions Biol. Syst.* 35:67–145.
- Byers, B. R., and J. E. Arceneaux. 1998. Microbial iron transport: iron acquisition by pathogenic microorganisms. *Met. Ions Biol. Syst.* 35:37–66.
- Faulkner, A., and M. Peaker. 1982. Reviews of the progress of dairy science: secretion of citrate into milk. *J. Dairy Res.* 49:159–169.
- Frost, G. E., and H. Rosenberg. 1973. The inducible citrate-dependent iron transport system in *Escherichia coli* K12. *Biochim. Biophys. Acta.* 330:90–101.
- Guerinot, M. L. 1994. Microbial iron transport. *Annu. Rev. Microbiol.* 48:743–772.
- Harle, C., I. Kim, A. Angerer, and V. Braun. 1995. Signal transfer through three compartments: transcription initiation of the *Escherichia coli* ferric citrate transport system from the cell surface. *EMBO J.* 14:1430–1438.
- Hussein, S., K. Hantke, and V. Braun. 1981. Citrate-dependent iron transport system in *Escherichia coli* K–12. *Eur. J. Biochem.* 117:431–437.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.
- Lin, J., J. S. Hogan, and K. L. Smith. 1998. Inhibition of in vitro growth of coliform bacteria by a monoclonal antibody directed against ferric enterobactin receptor FepA. *J. Dairy Sci.* 81:1267–1274.
- Lin, J., J. S. Hogan, and K. L. Smith. 1999. Antigenic homology of the inducible ferric citrate receptor (FecA) of coliform bacteria isolated from herds with naturally occurring bovine intramammary infections. *Clin. Diagn. Lab. Immunol.* 6:966–969.
- Murphy, C. K., V. I. Kalve, and P. E. Klebba. 1990. Surface topology of the *Escherichia coli* K–12 ferric enterobactin receptor. *J. Bacteriol.* 172:2736–2746.
- Nikaido, H. 1994. Porins and specific diffusion channels in bacterial outer membranes. *J. Biol. Chem.* 269:3905–3908.
- SAS User's Guide: Statistics. 1999. SAS Inst. Inc. Cary, NC.
- Takemura, K., J. S. Hogan, J. Lin, and K. L. Smith. 2002. Efficacy of immunization with ferric citrate receptor FecA from *Escherichia coli* on induced coliform mastitis. *J. Dairy Sci.* 85:774–781.
- Todhunter, D. A., K. L. Smith, and J. S. Hogan. 1991. Antibodies to iron-regulated outer membrane proteins of coliform bacteria isolated from bovine intramammary infections. *Vet. Immunol. Immunopathol.* 28:107–115.
- Van der Helm, D. 1998. The physical chemistry of bacterial outer-membrane siderophore receptor proteins. *Met. Ions Biol. Syst.* 35:355–401.
- Van Hove, B., H. Staudenmaier, and V. Braun. 1990. Novel two-component transmembrane transcription control: regulation of iron dicitrate transport in *Escherichia coli* K–12. *J. Bacteriol.* 172:6749–6758.
- Wagegg, W., and V. Braun. 1981. Ferric citrate transport in *Escherichia coli* requires outer membrane receptor protein fecA. *J. Bacteriol.* 145:156–163.
- Weinberg, E. D. 1978. Iron and infection. *Microbiol. Rev.* 42:45–66.