J. Dairy Sci. 85:1454–1459

Phagocytosis and Serum Susceptibility of *Escherichia coli* Cultured in Iron-Deplete and Iron-Replete Media[1]

A. J. Wise, J. S. Hogan, V. B. Cannon, and K. L. Smith
Department of Animal Sciences
The Ohio State University
Ohio Agricultural Research and Development Center, Wooster 44691

ABSTRACT

The susceptibility of *Escherichia coli* cultured in either iron-deplete or iron-replete media to phagocytosis by bovine neutrophils and the bactericidal activity of bovine serum was tested in vitro. Fourteen *E. coli* isolates from naturally occurring intramammary infections (IMI) were cultured overnight at 37°C in iron-replete media and iron-deplete media. The iron-replete media were trypticase soy broth or a chemically defined medium. The iron-deplete media were either trypticase soy broth plus 0.2 m*M* α, α' dipyridyl and 1m*M* citrate, or the chemically defined medium plus 0.2 m*M* α, α' dipyridyl, and 1 m*M* citrate. Iron-replenished medium was the chemically defined iron-deplete medium plus 40 m*M* ferric citrate. Bacteria grown in iron-deplete media were less susceptible to phagocytosis compared with bacteria grown in iron-replete media. Replenishing the chemically defined iron-deplete medium with ferric citrate obliterated the decreased susceptibility to phagocytosis observed in iron-deplete media. The iron availability in media used to culture *E. coli* before assay did not affect the bactericidal action of either the classical pathway of complement or the antibody independent alternative pathway of complement in serum. The growth of bacteria in iron-deplete medium did not alter the expression of capsule compared with growth in iron-replete medium. Iron availability during culture of *E. coli* altered the susceptibility of isolates to phagocytosis by neutrophils, but had no effect on the susceptibility of isolates to the bactericidal activity of serum.
(**Key words:** *Escherichia coli*, iron, phagocytosis)

Abbreviation key: EGTA = ethyleneglycol-bis-(aminoethyl ether) N, N, N′, N′ tetracetic acid, **HBSS** = Hank's balanced salt solution, **TSB** = trypticase soy broth.

INTRODUCTION

Virulence factors ascribed to *Escherichia coli* isolated from bovine IMI have included resistance to phagocytosis by neutrophils and resistance to the bactericidal actions of serum. The severity of clinical mastitis and duration of IMI caused by *E. coli* during lactation were correlated with the ability of isolates to avoid phagocytosis (Hill et al., 1983; Hogan et al., 1992). Carroll (1973) reported that serum susceptibility of *E. coli* strains was correlated with both pathogenicity and virulence of experimentally induced mastitis. A common aspect between the classical pathway of serum-mediated killing and the phagocytosis of *E. coli* is that antibody binding to specific surface antigens initiates both processes. Antibody binding to cell surfaces serves as opsonin for phagocytosis and activation of complement for serum killing of *E. coli*.

Iron is essential to *E. coli* but is not freely available to gram-negative bacteria in mammalian tissue (Chart and Griffiths, 1985; Bullen et al., 1978). Free iron in bovine milk is limited because iron is primarily bound to citrate and, to a lesser degree, to lactoferrin, transferrin, xanthine oxidase, and some caseins (Jenness, 1974). Therefore, the bovine mammary gland is an iron-restricted environment for coliform bacteria. The antigens exposed on the outer membranes of *E. coli* differ when isolates are cultured in iron-deplete and iron-replete media (Lin et al., 1999). Expression of iron-regulated outer-membrane proteins by *E. coli* in iron-deficient synthetic media and whey altered the binding of immunoglobulin to bacteria (Todhunter et al., 1990). Data are limited on the effects of iron availability in media on expression of virulence and pathogenicity factors commonly associated with *E. coli* infecting bovine mammary glands. The purpose of the current study was to compare the susceptibility of *E. coli* cultured in iron-deplete and iron-replete media to phagocytosis by bovine neutrophils and bactericidal activity of bovine serum.

MATERIALS AND METHODS

Isolates Cultured in Trypticase Soy Broth

Bacterial isolates. Fourteen isolates of *E. coli* from naturally occurring bovine IMI were used in the study.

Received July 9, 2001.
Accepted January 9, 2002.
Corresponding author: J. S. Hogan; e-mail: hogan.4@osu.edu.
[1]Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural and Research Development Center, The Ohio State University (17-01AS).

Isolates were grown overnight in iron-replete medium and iron-deplete medium at 37°C and 50 rpm on a gyratory shaker. Iron-replete medium was trypticase soy broth (**TSB**; Becton Dickinson Microbiology System, Sparks, MD). Iron-deplete medium was TSB plus 0.2 m*M* α, α' dipyridyl (Sigma Chemical Co., St. Louis, MO), and 1m*M* citrate (Sigma Chemical Co.). The bacteria were pelleted by centrifugation and resuspended in Hanks balanced salts solution (**HBSS**; Sigma Chemical Co.) to 40×10^6 cfu/ml. Bacteria were plated on MacConkey agar (Becton Dickinson Microbiology System, Sparks, MD) and incubated 15 h at 37°C to confirm counts.

Serum. Serum used in the study was pooled from the blood of nine nonlactating cows in the Ohio Agricultural Research and Development Center dairy herd. Approximately one-half of the pooled serum sample was heat-inactivated at 56°C for 30 min. Normal and heat-inactivated sera were stored as 1-ml samples at –20°C.

Preparation of blood neutrophils. Blood was collected from the jugular veins of two cows (50 ml each) on a daily basis. Neutrophils were isolated according to Carlson and Kaneko (1973). Neutrophil viability was measured by trypan blue exclusion. The total number of cells was determined with the aid of a hemocytometer. A differential stain (Diff-Quick; Baxter Healthcare Cooperation, Miami, FL) was also done to determine the total percentage of neutrophils. The number of viable neutrophils was determined as [(neutrophil viability) (the total number of cells) (the percentage of neutrophils)]. Cell preparations averaged (mean ± SD) 99.4 ± 0.7% neutrophils and 95.4 ± 1.8% viability. Viable neutrophil concentrations were adjusted to 40×10^6 viable neutrophils/ml of HBSS.

Opsonization. Bacteria were opsonized with 10% heat-inactivated serum for 20 min at 20°C before use in neutrophil assays.

Phagocytic assay. Assays were prepared as a 2:1 bacteria:neutrophils ratio. One milliliter of opsonized bacteria and 500 μl of neutrophils were mixed together in sterile 12 × 75-mm culture tubes. The assays were incubated for 1.5 h at 37°C and 100 rpm on a gyratory shaker. After incubation, the samples were diluted 2:1:1 as 50 μl of assay suspension: 25 μl of acridine orange (1.4 mg per 10 ml of PBS): 25 μl of crystal violet (5 mg per 10 ml of PBS). Wet mount slides were prepared, and bacterial cells were counted in the first 25 neutrophils visible under the 1000X oil-immersion lens as the microscope stage (Nikon Fluorescence Microscope, Garden City, NY) was moved from left to right on the cover slip. A bacterial isolate was tested after culture in both media on the same day to reduce interassay variability between treatments. The assays were in duplicate and replicated on separate days. The person reading and recording phagocytic results was blinded relative to the bacterial isolates and growth conditions used in assays. Variables measured were phagocytic index (number of intracellular bacteria/total number of neutrophils), the percentage of neutrophils phagocytizing (number of neutrophils with intracellular bacteria/total number of neutrophils) and the number of bacteria per positive neutrophil (number of intracellular bacteria/number of positive neutrophils). Positive neutrophils were defined as those with one or more intracellular bacteria.

Serum susceptibility assays. The effects of serum, heat-inactivated serum, and the alternative pathway of complement on bacterial growth responses of isolates cultured in iron-replete and iron-deplete media were measured in 96-well microtiter plates. Each well had a capacity of 300 μl. Aliquots of 125 μl were allotted to either normal or heat-inactivated serum, 100 μl to Tris-NaCl buffer, and 25 μl to the bacterial inoculum. Bacterial inoculum of assays was approximately 40×10^6 cfu/ml. The classical pathway of complement was inhibited by the addition of both ethylene glycol-bis-(aminoethyl ether) N, N, N′, N′ tetracetic acid (**EGTA**; Sigma Chemical Co.) and $MgCl_2$ (Sigma Chemical Co.) to serum for a final assay concentration of 43 m*M* (MacDonald et al., 1983). Both EGTA and $MgCl_2$ were dissolved in Tris-$NaCl_2$ buffer before addition to assay wells.

Assay plates were incubated and viable bacteria were determined after 0, 4, and 24 h of incubation at 37°C. Bacteria were plated on MacConkey agar and incubated 15 h at 37°C. Bacterial counts were expressed as colony-forming units $\log_{10}$/ml.

Capsule staining. Bacterial isolates cultured in iron-replete and iron-deplete media were stained for the presence of a capsule. Cellular capsules were detected by the India ink (Faber-Castell Co., Newark, NJ) staining procedure. Ten microliters of culture and 10 μl of India ink were mixed, smeared on a microscope slide, and air dried. Bacteria were counterstained with gentian violet (Harleco Stains, Gibbstown, NJ). Isolates with a clear zone of greater than two micrometers were considered to be encapsulated.

Statistical analysis. Phagocytic data were analyzed using least squares analyses of variance with data blocked by isolate (SAS, 1999). Models included the main effects of iron culture condition, presence or absence of cellular capsule, and the interaction between iron culture condition and capsule. Susceptibility to the bactericidal activity of serum was tested using least squares ANOVA (SAS, 1999). Models for serum susceptibility data included main effects of iron culture condition, time of incubation, and interactions between iron culture condition and time of incubation.

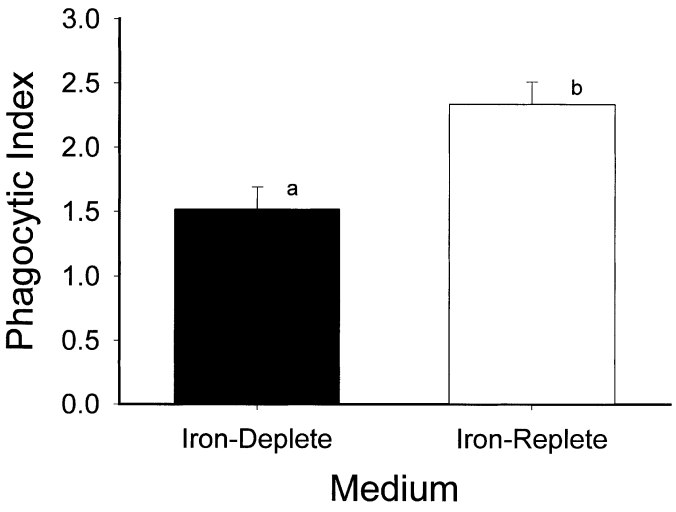

Figure 1. The phagocytic index of *Escherichia coli* isolates (n = 14) cultured in iron-deplete medium (solid bar) and iron-replete medium (empty bar). Dispersion bars represent standard errors for each growth condition. [a,b]Means between growth conditions with differing letters are different ($P \leq 0.01$).

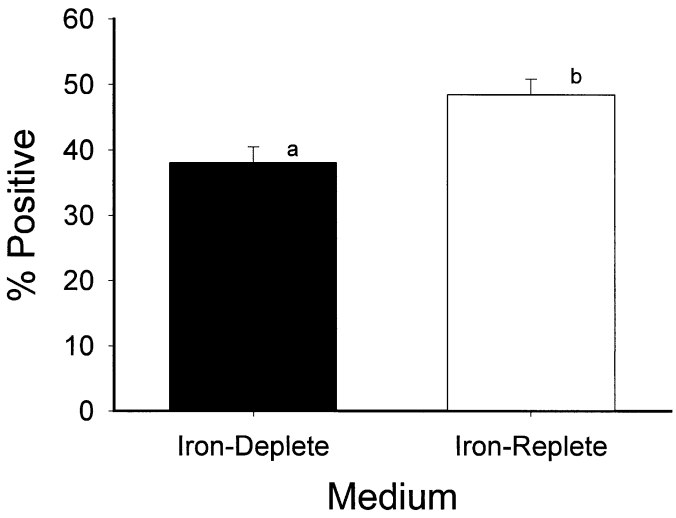

Figure 2. The percentage of neutrophils phagocytizing (% positive) *Escherichia coli* isolates (n = 14) cultured in iron-deplete medium (solid bar) and iron-replete medium (empty bar). Dispersion bars represent standard errors for each growth condition. [a,b]Means between growth conditions with differing letters are different ($P \leq 0.05$).

Isolates Cultured in Chemically Defined Media

The influence of supplementing iron-deplete media with exogenous iron on the susceptibility of *E. coli* to phagocytosis was tested using a chemically defined medium (Bacto-synthetic broth). *Escherichia coli* were cultured before phagocytosis assays were performed in either the chemically defined iron-replete medium, a chemically defined iron-deplete medium (chemically defined medium plus 0.2 m*M* α, α' dipyridyl and 1 m*M* citrate), and chemically defined iron-replenished media (chemically defined iron-deplete medium plus 40 m*M* ferric citrate). Bacterial strains tested, incubation times, and temperatures were as described previously for culturing bacteria in TSB. Blood neutrophils were collected and prepared as described above. Cell preparations averaged (mean ± SD) 98.6 ± 1.6% neutrophils and 99.8 ± 0.4% viability. Opsonization of bacteria and phagocytic assays were performed as described above.

Phagocytic data were analyzed by least squares ANOVA with data blocked by isolate (SAS, 1999). Differences among means were tested by Tukey's multiple comparison test.

RESULTS

Isolates Cultured in Trypticase Soy Broth

Phagocytosis. Bacteria grown in iron-deplete TSB consistently had a lower phagocytic index ($P \leq 0.01$ [Figure 1]), a reduced percentage of neutrophils phagocytizing bacteria ($P \leq 0.05$ [Figure 2]), and fewer bacteria per positive neutrophil ($P \leq 0.05$ [Figure 3]) compared with bacteria grown in iron-replete TSB. Although the phagocytic index, the percentage of neutrophils phagocytizing, and the number of bacteria per positive neutrophil varied greatly among isolates, phagocytic parameters were reduced for each of the 14 isolates when cultured in iron-deplete TSB. The growth

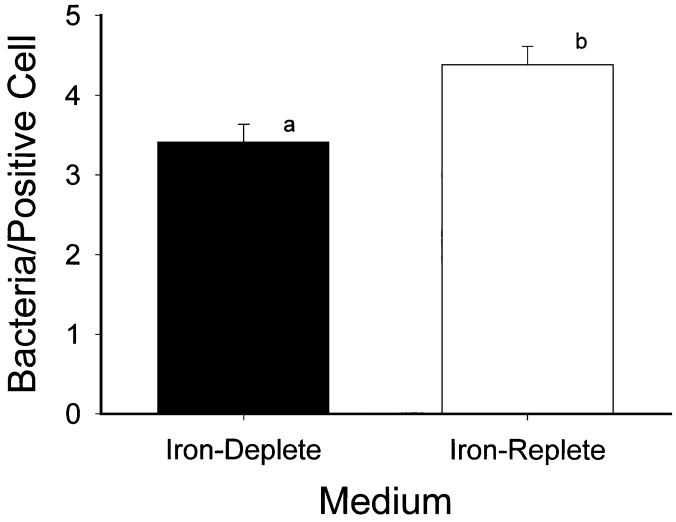

Figure 3. The number of bacteria per phagocytizing neutrophil for *Escherichia coli* isolates (n = 14) cultured in iron-deplete medium (solid bar) and iron-replete medium (empty bar). Dispersion bars represent standard errors for each growth condition. [a,b]Means between growth conditions with differing letters are different ($P \leq 0.05$).

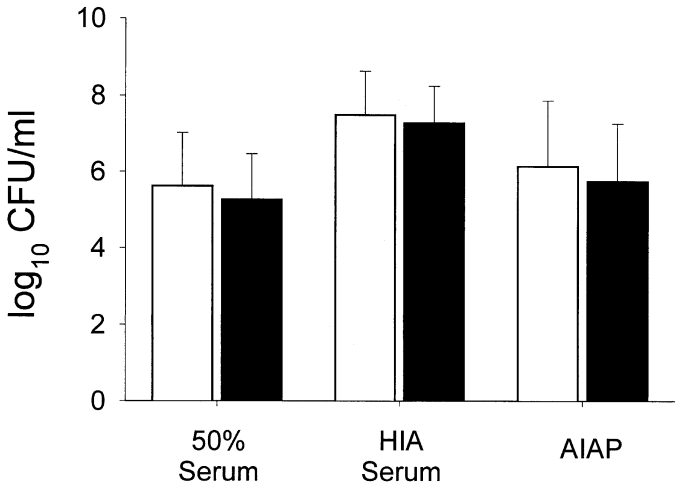

Figure 4. Growth responses of *Escherichia coli* isolates (n = 14) cultured in iron-deplete medium (solid bar) and iron-replete medium (empty bar) to bovine serum, heat-inactivated serum (HIA), and the antibody-independent alternative pathway of complement (AIAP) after a 4-h incubation. Dispersion bars represent standard errors for each growth condition.

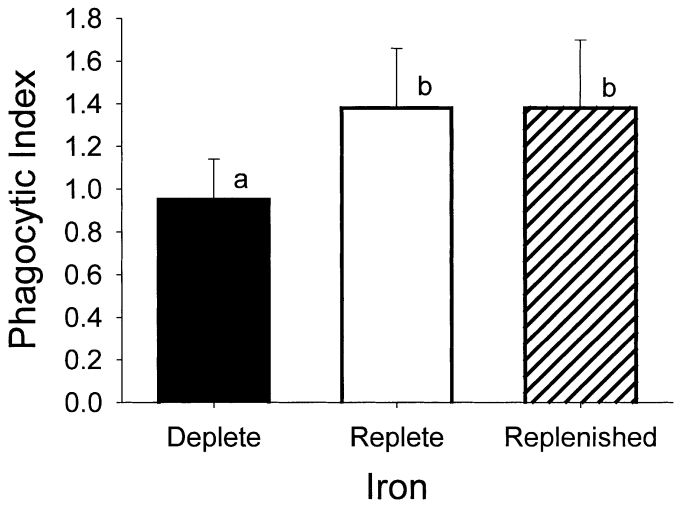

Figure 6. The phagocytic index of *Escherichia coli* isolates (n = 14) cultured in iron-deplete chemically defined medium (solid bar), iron-replete chemically defined medium (empty bar), and iron-replenished chemically defined medium (hashed bar). Dispersion bars represent standard errors for each growth condition. [a,b]Means between growth conditions with differing letters are different ($P \leq 0.05$).

of *E. coli* isolates in iron-deplete TSB decreased the phagocytic index by an average of 40% (range 18 to 68%) compared with growth in iron-replete TSB.

Serum susceptibility. Serum susceptibility to 50% pooled serum did not differ ($P > 0.05$) between isolates cultured in iron-deplete TSB and iron-replete TSB after 4 h (Figure 4) and 24 h (Figure 5) of incubation. The iron availability in media used to culture *E. coli* before assay did not affect the bactericidal action of the antibody-independent alternative pathway of complement in serum ($P > 0.05$). Bacterial growth in heat-inactivated serum after a 4- and 24-h incubation was similar between isolates cultured in iron-deplete TSB and iron-replete TSB ($P > 0.05$).

Capsule. The growth of bacteria in iron-deplete TSB did not alter the expression of capsule compared with growth in iron-replete TSB. A capsule was observed on three *E. coli* isolates in both culture media. Furthermore, phagocytic parameters did not differ between encapsulated and nonencapsulated *E. coli* isolates ($P > 0.05$). The mean (± SE) phagocytic index, percentage of neutrophils phagocytizing bacteria, and number of bacteria per positive neutrophil for encapsulated and nonencapsulated strains were 1.8 ± 0.3 and 2.1 ± 0.6, 43.6 ± 3.1 and 42.0 ± 8.7, and 3.8 ± 0.3 and 4.1 ± 0.8, respectively.

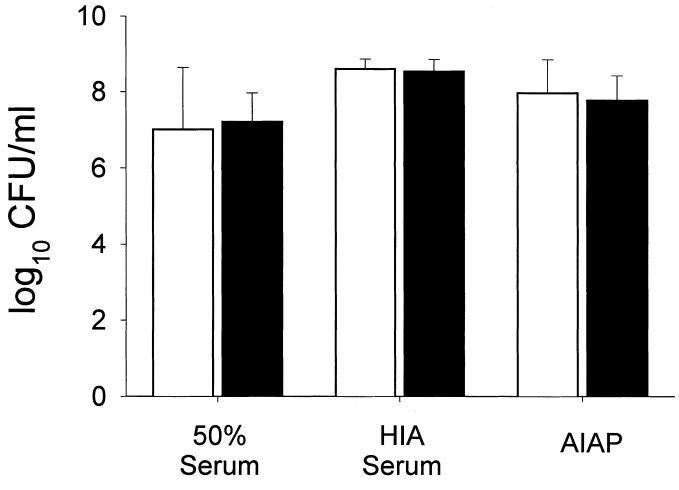

Figure 5. Growth responses of *Escherichia coli* isolates (n = 14) cultured in iron-deplete medium (solid bar) and iron-replete medium (empty bar) to bovine serum, heat-inactivated serum (HIA), and the antibody-independent alternative pathway of complement (AIAP) after a 24-h incubation. Dispersion bars represent standard errors for each growth condition.

Isolates Cultured in Chemically Defined Media

Bacteria grown in iron-deplete chemically defined medium had a lower phagocytic index ($P \leq 0.05$; Figure 6) and fewer bacteria per positive neutrophil ($P \leq 0.05$; Figure 7) compared with bacteria grown in iron-replete chemically defined media. Replenishing iron-deplete chemically defined medium with 40 m*M* ferric citrate resulted in a higher phagocytic index and greater number of bacteria per positive neutrophil than for bacteria cultured in the chemically defined iron-deplete medium ($P \leq 0.05$). Phagocytosis data were comparable among

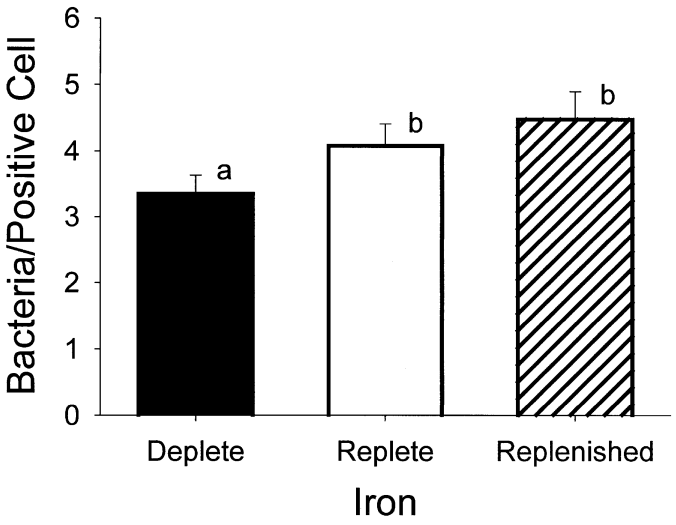

Figure 7. The number of bacteria per phagocytizing neutrophil for *Escherichia coli* isolates (n = 14) cultured in iron-deplete chemically defined medium (solid bar), iron-replete chemically defined medium (empty bar), and iron-replenished chemically defined medium (hashed bar). Dispersion bars represent standard errors for each growth condition. [a,b]Means between growth conditions with differing letters are different ($P \leq 0.05$).

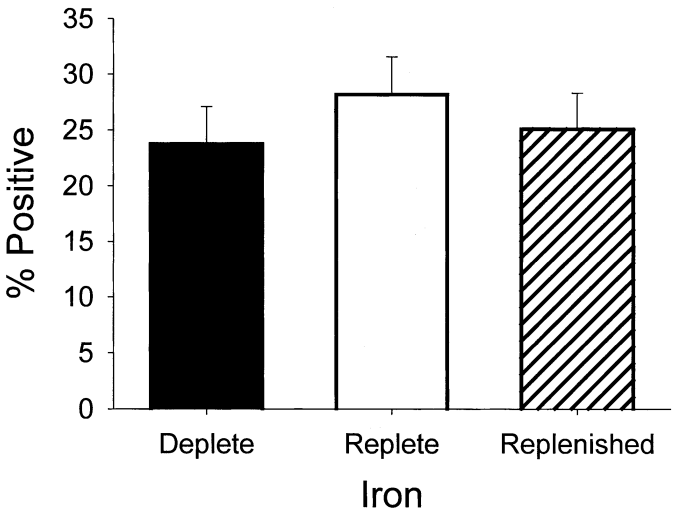

Figure 8. The percentage of neutrophils phagocytizing (% positive) of *Escherichia coli* isolates (n = 14) cultured in iron-deplete chemically defined medium (solid bar), iron-replete chemically defined medium (empty bar), and iron-replenished chemically defined medium (hashed bar). Dispersion bars represent standard errors for each growth condition.

bacteria cultured in chemically defined iron-replete medium and chemically defined iron-replenished medium ($P \geq 0.05$). Percentage of neutrophils phagocytizing (Figure 8) did not differ among treatments ($P \geq 0.05$).

DISCUSSION

The iron availability in medium used to culture *E. coli* prior to assay altered the susceptibility of isolates to phagocytosis by neutrophils. The phagocytic parameters for *E. coli* cultured in iron-deplete media were reduced compared with the phagocytosis of isolates cultured in iron-replete media. Furthermore, in the initial trial in TSB, the reduction in phagocytosis was consistent among all isolates tested. Bacteria were subsequently tested in a chemically defined medium to investigate if the reduction in phagocytosis would be repeated in different media. The reduction in susceptibility to phagocytosis was comparable between media. In addition, replenishing the chemically defined iron-deplete medium with ferric citrate obliterated the decreased susceptibility to phagocytosis. Previous studies have demonstrated a wide range of susceptibility to phagocytosis among *E. coli* isolated from naturally occurring bovine IMI. The variability among isolates was attributed to strain differences in opsonization mediated by antibodies directed against surface-exposed antigens (Hogan et al., 1992). The variability in phagocytic parameters observed within isolates in the present study may be due to the alteration of surface-exposed antigens in the iron-deplete conditions. Changes in cell surfaces of *Staphylococcus aureus* cultured in iron-deplete medium resulted in reduction of phagocytic parameters by human neutrophils (Domingue et al., 1989). Growth of bacteria in iron-deplete media alters the expression of outer-membrane proteins (Chart et al., 1986). Lin et al. (1999) reported that *E. coli* isolates cultured in synthetic media containing the iron chelator, α, α' dipyridyl and 1 m*M* citrate, expressed outer-membrane proteins with antigenicity similar to *E. coli* cultured in milk. However, the relationship between the decreased susceptibility to phagocytosis of *E. coli* observed in vitro and the iron-restricted environment within the bovine mammary gland is speculative. Previous trials have shown positive relationships between the ability of *E. coli* strains to evade in vitro phagocytosis and the duration and severity of naturally occurring IMI from which strains were isolated (Hill et al., 1981; Hogan et al., 1992).

The ability of *E. coli* to evade phagocytosis by neutrophils and cause mastitis during lactation was ascribed to capsule formation (Hill et al., 1983). The growth of bacteria in iron-deplete TSB did not stimulate a capsule to be expressed in isolates that were capsule-negative when cultured in iron-replete TSB. A capsule was expressed in three *E. coli* isolates grown in both iron-deplete and iron-replete TSB. Phagocytosis was hindered by the presence of a capsule on *E. coli* (Hill et al., 1983). The lack of effect of capsule on phagocytosis

in the current study may have been due to the relatively small percentage of encapsulated *E. coli* isolates tested.

The iron availability in medium used to culture *E. coli* before assay did not alter the susceptibility of isolates to the bactericidal activity of bovine serum. The bactericidal capacity of bovine serum was due to complement activity (Hogan et al., 1989). Heat inactivation of complement rendered the serum benign to all isolates in the current study. The efficiency of complement binding to the bacterial surface of different isolates was dependent on the differences between bacterial surfaces (Clas and Loos, 1980). The serum susceptibility of *E. coli* was altered by changes in culture conditions which resulted in an increase in outer-membrane permeability (Ocana-Morgner and Dankert, 2001). In contrast, the differences in cellular surfaces induced by iron-limited medium did not change the susceptibility to the bactericidal actions of complement in the current study. Both the classical and alternative pathways of complement are important in serum killing of *E. coli* (Korhonen et al., 2000). The classical pathway is activated by the binding of antibody to cell-surface antigens (Schrieber et al., 1979). The changes in antigen expression previously shown to be induced by iron depletion (Lin et al., 1999) did not influence the killing of isolates by the classical pathway of complement in the current study. Therefore, the alterations in cell surfaces were not critical to limiting the binding of antibody sufficient to activate complement in the assay serum. The alternative pathway of complement is independent of antibody (Schrieber et al., 1979), thus less reliant on conservation of surface antigens on bacteria for activation and lysis of bacteria. Although the alternative pathway accounted for the greatest reduction in bacterial growth, neither pathway was altered by iron availability to isolates.

CONCLUSIONS

The growth of *E. coli* in iron-deplete media decreased the susceptibility of bacteria to phagocytosis by bovine neutrophils. In addition to iron acquisition, the expression of iron-regulated outer-membrane proteins and other cell-surface changes may enhance the pathogenicity of *E. coli* by rendering the bacteria less susceptible to phagocytosis. However, the susceptibility of *E. coli* to the bactericidal activity of serum and the expression of capsule were not responsive to the availability of iron during culture.

REFERENCES

Bullen, J. J., H. J. Rogers, and E. Griffiths. 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. 80:1–35.

Carlson, G. P., and J. J. Kaneko. 1973. Isolation of leukocytes from bovine peripheral blood. Proc. Soc. Exp. Biol. Med. 142:853–856.

Carroll, E. J. 1973. Experimentally induced coliform mastitis: Inoculation of udders with serum-sensitive and serum-resistant organisms. Am. J. Vet. Res. 34:1143–1146.

Chart, H., and E. Griffiths. 1985. Antigenic and molecular homology of the ferric enterobactin receptor protein of *Escherichia coli*. J. Gen. Microbiol. 131:1503–1509.

Clas, F., and M. Loos. 1980. Killing of the S and Re forms of *Salmonella minnesota* via the classical activation in guinea-pig and human sera. Immunology 40:547–556.

Domingue, P. A. G., P. A. Lambert, and M. R. W. Brown. 1989. Iron depletion alters surface-associated properties of *Staphylococcus aureus* and its association to human neutrophils in chemiluminescence. FEMS Microbiol. Lett. 59:265–268.

Hill, A. W. 1981. Factors influencing the outcome of *Escherichia coli* mastitis in the dairy cow. Res. Vet. Sci. 31:107–112.

Hill, A. W., D. J. S. Heneghan, and M. R. Williams. 1983. The opsonic activity of bovine whey for the phagocytosis and killing by neutrophils of encapsulated and non-encapsulated *Escherichia coli*. Vet. Microbiol. 8:293–300.

Hogan, J. S., D. A. Todhunter, K. L. Smith, and P. S. Schoenberger. 1992. Susceptibility of *Escherichia coli* isolated from intramammary infections to phagocytosis by bovine neutrophils. J. Dairy Sci. 75:3324–3329.

Hogan, J. S., D. A. Todhunter, K. L. Smith, and P. S. Schoenberger. 1989. Serum susceptibility of coliforms from bovine intramammary infections. J. Dairy Sci. 72:1893–1899.

Jenness, R. 1974. The composition of milk. Pages 3–107 in Lactation: A Comprehensive Treatise. Vol III. B. L. Larson and V. R. Smith, ed. Academic Press, New York, NY.

Korhonen, H., P. Marnila, and H. S. Gill. 2000. Milk immunoglobulins and complement factors. Br. J. Nutr. 84:S75–S80.

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.

MacDonald, J. T., S. K. Maheswaran, J. Opuda-Asibo, E. L. Townsend, and E. S. Thies. 1983. Susceptibility of *Pasteurella haemolytica* to the effects of serum, nasal secretions and bronchoalveolar washing from cattle. Vet. Microbiol. 8:585-599.

Ocana-Morgner, C., and J. R. Dankert. 2001. Induction of complement sensitivity in *Escherichia coli* by citric acid and low pH. J. Appl. Microbiol. 90:771–778.

SAS User's Guide: Statistics, Version 8 Edition. 1999. SAS Inst., Inc., Cary, NC.

Schrieber, R. D., D. C. Morrison, E. R. Podack, and H. J. Muller-Eberhard. 1979. Bactericidal activity of the alternative complement pathway generated from 11 isolated plasma proteins. J. Exp. Med. 149:870–882.

Todhunter, D. A., K. L. Smith, and J. S. Hogan. 1990. Antibodies to iron-regulated outer membrane proteins of coliforms isolated from bovine intramammary infections. Vet. Immun. Immunopathol. 28:107–115.