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In Vitro Attachment and Invasion of Chicken Ovarian Granulosa Cells by *Salmonella enteritidis* Phage Type 8

DORAIRAJAN THIAGARAJAN,[1] MAHDI SAEED,[1]* JOHN TUREK,[2] AND ELIKPLIMI ASEM[2]

Department of Veterinary Pathobiology,[1] and Department of Basic Medical Sciences,[2] School of Veterinary Medicine, Purdue University, West Lafayette, Indiana 47907

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The attachment and invasion of chicken ovarian granulosa cells by *Salmonella enteritidis* was examined in vitro. The attachment was inhibited by preincubation of granulosa cells with anti-chicken fibronectin antibody (approximately 70% reduction in attachment) or preincubation with a 14-kDa fimbrial protein isolated from *S. enteritidis* (68% reduction in attachment). Treatment of bacterial cells with the tetrapeptide RGDS before addition to granulosa cells resulted in inhibition of attachment (60% inhibition when 2×10^7 CFU of bacteria was treated with 500 μg of peptide). Treatment with the peptide GRGD resulted in similar magnitude of inhibition, indicating that extracellular matrix proteins play significant roles in the interaction of *S. enteritidis* with granulosa cells. In contrast, treatment of the bacterial cells with the peptide GRAD did not result in significant inhibition of attachment to the granulosa cells. *S. enteritidis* was found to attach specifically to fibronectin, collagen IV, and laminin-coated microtiter plate wells, with the rank order of attachment as follows: fibronectin > laminin > collagen IV. Light and transmission electron micrographs of *S. enteritidis* invasion of granulosa cells showed organisms with or without a surrounding membrane in the cytoplasm of granulosa cells. In some instances, dividing bacterial cells were observed in the cytoplasm. Results of this study demonstrated that *S. enteritidis* interacts with granulosa cells in a specific manner and can invade and multiply in these cells. The granulosa cell layer of the preovulatory follicles may be a preferred site for the colonization of the chicken ovaries by invasive strains of *S. enteritidis*.

The notion that grade A shell eggs can act as vehicles for food poisoning by *Salmonella enteritidis* has gained ample significance during the past several years (8–10, 14, 22, 25, 27). Contamination of eggs can occur either by shell surface contamination with fecal matter or through yolk contamination as the egg is formed in vivo. It has been proposed that the majority of egg contamination in the United States may occur through in vivo contamination because the outer shell surface is sanitized thoroughly prior to shipping of eggs to retail markets (34). To understand the pathogenesis of *S. enteritidis* infection in chickens, investigators have studied the consequences following experimental, oral inoculations of chickens with *S. enteritidis* (5, 6, 15, 16, 33, 36). Various phage types and strains of *S. enteritidis* differ in their virulence to chickens (19, 20). However, the microbial characteristics and the processes that lead to egg contamination in vivo have not been elucidated.

Attachment of bacteria to host cell surface may be an essential step in pathogenesis (13). This interaction probably involves surface structures on the bacteria that are capable of binding membrane component(s) on the host cell surface. For example, adhesion of *S. enteritidis* to mouse intestinal epithelial cells requires two types of fimbriae and is viewed as an important initial step in the pathogenesis of the disease in mice (1). In other studies, the adhesion of *S. typhimurium* and *S. enteritidis* to human intestinal cells was shown to be mediated by fimbriae, and fibronectin was shown to be involved in the binding process (4). *Escherichia coli* and *S. enteritidis* are capable of binding other extracellular matrix proteins such as laminin and collagens as well (23). In light of these and other findings, it was proposed that the binding of extracellular matrix proteins by microorganisms may be a mechanism by which tissue adherence occurs and thus may be a significant step in disease pathogenesis (21).

Following experimental oral inoculation of laying hens with *S. enteritidis*, the organism was isolated from the tissue layers surrounding the yolk in preovulatory follicles (15, 35). This finding indicates that the bacteria may interact with the cellular component(s) of the preovulatory follicle. *S. enteritidis* isolates of various phage types have been shown to attach to chicken ovarian granulosa cells that were isolated from healthy, adult, laying hens and cultured in vitro (35). The tetrapeptide sequence Arg-Gly-Asp-Ser (RGDS) of a number of extracellular matrix proteins plays an important role in the attachment and binding of these proteins to cell surfaces and macromolecules (30, 31, 37). It was shown that the synthetic peptide RGDS abrogated the attachment of *S. enteritidis* to granulosa cells (35). This finding suggested that cell surface-associated extracellular matrix proteins are important for the attachment of the bacteria to granulosa cells. However, the extent of the inhibition of attachment was not quantified. In addition, the roles of various bacterial surface structures in the attachment process were not examined.

The present study examined (i) the role of a 14-kDa *S. enteritidis* fimbrial protein in the attachment of *S. enteritidis* to chicken ovarian granulosa cells; (ii) the role of extracellular matrix proteins such as fibronectin, laminin, and collagen IV in the process; and (iii) the invasion of chicken ovarian granulosa cells in vitro by *S. enteritidis*.

MATERIALS AND METHODS

Hens. Single Comb White Leghorn hens (25 to 30 weeks old) were obtained from a local farm known to be free of *S. enteritidis* infections by serological testing. This was further confirmed by killing randomly selected birds and culturing visceral organs (ceca, liver, spleen, and ovaries) for *S. enteritidis* isolation. The birds were housed in individual wire mesh cages in a windowless, air-

* Corresponding author. Mailing address: Department of Veterinary Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907. Phone: (317) 494-5796. Fax: (317) 494-9830.

conditioned room with 16 h light/8 h darkness cycle. They had unlimited access to tap water and a commercial layer ration.

Bacterial strain. The bacterial strain used in this study (designated AE9) was *S. enteritidis* phage type 8, originally isolated from an outbreak of food poisoning due to contaminated eggs. It was stored at −70°C in tryptic soy broth containing 15% (vol/vol) glycerol. It is pathogenic to laying hens, as demonstrated by invasion of various internal organs, including preovulatory follicles, and isolation from a small proportion of laid eggs following oral inoculation of a group of White Leghorn chickens (35).

Radiolabeling of bacteria. For attachment inhibition assays, the bacterial cells were radiolabeled by cultivation in colonization factor antigen broth containing 80 μCi of $^{-14}$C in the form of sodium acetate. Specific activity per CFU ($1 \times$ to 2×10^{-2} cpm) was established by viable counts and measurement of radioactivity after harvest and two washes of bacterial cells in medium 199.

Purification of 14-kDa fimbrial protein. The 14-kDa fimbrial protein was purified as described previously (12), with few modifications. Briefly, *S. enteritidis* AE9 was grown in 10 liters of colonization factor antigen broth as static, aerobic cultures for 48 h. The bacterial culture was centrifuged at $3,000 \times g$ for 15 min at 10°C and resuspended in 80 ml of 0.15 M ethanolamine buffer (pH 10.5). The fimbriae were separated from the bacterial cells by shearing for 2 min in a laboratory blender. The process was repeated five times at 2-min intervals. After the bacterial cells and cellular debris were removed by centrifugation at $3,000 \times g$ for 15 min at 10°C, ammonium sulfate was added to the supernatant to 10% saturation and left overnight at 4°C with constant stirring. The mixture was centrifuged at $10,000 \times g$ for 15 min. Ammonium sulfate was added to the supernatant to 40% saturation and left overnight with constant stirring. The precipitated proteins were pelleted by centrifugation at $10,000 \times g$ for 15 min. The pellet was then suspended in 0.15 M ethanolamine buffer (pH 10.5) and centrifuged at $115,000 \times g$ for 1 h at 4°C. The precipitate obtained after centrifugation was resuspended in 0.15 M ethanolamine buffer and dialyzed overnight against 0.02 M Tris-HCl followed by extensive dialysis against distilled water. Protein determinations were done by the bicinchoninic acid method (Pierce Chemical Company, Rockford, Ill.). Bovine serum albumin (BSA) was used as the standard protein. The protein preparations were lyophilized and stored at −20°C until further use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation of proteins. To analyze fimbrial protein preparations, a 16- by 14-cm vertical slab gel system (4.5% stacking gel and 15% separation gel) was used. The protein preparations were solubilized in Laemmli buffer (24) and electrophoresed along with molecular weight standards. The separated protein bands were stained with 0.025% Coomassie brilliant blue.

Amino acid composition analysis of 14-kDa fimbrial protein. The purified protein was hydrolyzed by using 6 N HCl, and the amino acid composition was analyzed with a Beckman amino acid analyzer (Beckman Instruments Inc., Fullerton, Calif.). BSA was used as control to determine the efficiency of hydrolysis and the analytical process.

Granulosa cell preparation. The granulosa layers of the ovarian tissue of healthy adult laying hens were separated from the thecal layers as previously described (17), and the cells were dissociated in collagenase (Sigma Chemical Co., St. Louis, Mo.) containing medium 199 (Life Technologies Corp., Gaithersburg, Md.). The dispersed cells were suspended in medium 199 supplemented with 0.2% BSA, 100 U of penicillin G per ml, and 100 μg of streptomycin sulfate per ml (29).

In vitro attachment inhibition assays. Chicken ovarian granulosa cells (10^5 per well) were cultured overnight at 37°C under 5% CO_2 atmosphere in 96-well tissue culture plates. ^{14}C-labeled *S. enteritidis* AE9 was washed in medium 199 and added at a density of 10^7 CFU per well. In all the in vitro attachment inhibition assays described below, radiolabeled bacteria were added to tissue culture wells without granulosa cells, and the recovered radioactivity after 1 h of incubation at 37°C (background) was subtracted from the test wells.

For inhibition of attachment using rabbit anti-chicken fibronectin antibody (Chemicon International Corp., Temecula, Calif.), granulosa cells were incubated for 30 min at 37°C with various dilutions (10^{-4}-, 10^{-3}-, 10^{-2}-, and 10^{-1}-fold) of antibody before addition of radiolabeled bacteria. Each dilution was tested in triplicate. Control wells were incubated with 0.01% (wt/vol) rabbit gamma globulin (Sigma). Following incubation with bacteria for 1 h at 37°C, unbound bacteria were removed by five washes with Hanks' balanced salt solution (140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$, 5.6 mM glucose, 10 mM *N*-2-hydroxyethylpiperazine-*N*^{1}-2-ethanesulfonic acid [HEPES; pH 7.4]). The granulosa cells along with the attached bacteria were dissolved in 100 μl of 0.02 N sodium hydroxide. The mixture from each well was transferred to a separate scintillation vial containing scintillation fluid (Ecolite; ICN Pharmaceuticals, Costa Mesa, Calif.), and the radioactivity was measured in a Beckman scintillation counter. Radioactivity obtained from test wells was expressed as a percentage of the control value for each dilution of antibody tested. The radioactivity thus obtained is a measurement of the total number of bacteria associated with granulosa cells (extracellular bacteria plus intracellular bacteria).

In the case of inhibition experiments using 14-kDa fimbrial protein, the granulosa cells were incubated with various concentrations of the protein (0, 2.5, 5, 10, and 20 μg per well) for 1 h at 37°C before addition of radiolabeled bacteria. Control wells were incubated with medium 199 containing 0.1% (wt/vol) BSA. Attachment inhibition was measured as described above.

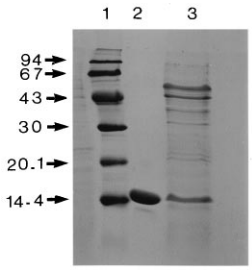

FIG. 1. SDS-PAGE profile of proteins isolated from *S. enteritidis* AE9. Lane 1, molecular weight markers; lane 2, purified fimbrial protein; lane 3, heat-extracted (65°C for 30 min) membrane-associated proteins. The numbers on the left indicate molecular masses of marker proteins in kilodaltons.

For attachment assays involving synthetic peptides, various concentrations of the peptides (125, 250, and 500 μg per ml) were incubated end-over-end with 2×10^7 CFU of bacteria per ml for 1 h at room temperature in the presence of 0.01% (vol/vol) Tween 80 (Sigma). At the end of the incubation, bacterial cells were washed with medium 199 containing 0.01% Tween and resuspended in the same medium before the granulosa cells were inoculated. Bacterial suspensions for control wells were incubated with 0.1% BSA and 0.01% Tween 80 in medium 199. Different concentrations for each peptide, RGDS (Sigma) and GRGD and GRAD (Life Technologies), were tested in triplicate. Radioactivity recovered from each test well was expressed as a percentage of the control value.

Solid-phase protein binding assay. Bovine plasma fibronectin (Sigma), mouse laminin, and mouse collagen type IV (Life Technologies), at various concentrations (0.5, 1, 2, and 4 μg per well) were applied to tissue culture wells in a 96-well plate (Corning, Fisher Scientific, Springfield, N.J.). The extracellular matrix proteins were dissolved in 50 μl of medium 199, transferred to microplate wells, and allowed to dry at room temperature. The efficiency of coating was tested by using specific antibodies against each of these proteins in an indirect enzyme-linked immunosorbent assay or by protein determination by the bicinchoninic acid method. For binding assays, *S. enteritidis* AE9 was inoculated (2×10^6 CFU per well) in the tissue culture wells and incubated for 1 h at 37°C. After the wells were washed five times with phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1 mM KH_2PO_4), the bound bacteria were recovered by using 0.02 N NaOH and the radioactivity was measured. Bacterial attachment to different concentrations of each extracellular matrix protein was tested in triplicate. Control wells in triplicate were coated with 1, 2, and 4 μg of BSA per well.

Invasion of granulosa cells by *S. enteritidis*. Invasion experiments were carried out essentially as described previously (28). Chicken ovarian granulosa cells were grown on glass coverslips in 24-well tissue culture plates (Corning, Fisher Scientific) at a concentration of 5×10^5 cells per ml. The cells were inoculated with 2×10^7 CFU of *S. enteritidis* AE9 grown overnight in beef heart infusion broth under static, aerobic conditions. After 2 h of incubation at 37°C, the cells were washed once with medium 199 and intracellular growth medium, which consists of medium 199 supplemented with 2% fetal bovine serum; 100 μg of gentamicin per ml was added, and the mixture was incubated at 37°C. During the incubation period, the medium was replaced every hour with intracellular medium (medium 199 containing 2% fetal bovine serum and 10 μg of gentamicin per ml). Coverslips were removed for examination after 3 h of incubation at 37°C. The coverslips were washed five times with PBS. For light microscopic observations, the coverslips were fixed with methanol and stained with 10% Giemsa stain. For electron microscopic observations, the coverslips were fixed with phosphate-buffered 3% glutaraldehyde solution.

Electron microscopy. The coverslips were further fixed with 1% osmium tetroxide–1.5% potassium ferrocyanide, dehydrated by passage through graded ethanol, rinsed twice in propylene oxide, and infiltrated with epoxy resin (Poly/Bed 812; Polysciences, Warrington, Pa.). The side of the coverslip containing the cells was inverted onto a polyethylene capsule filled with epoxy resin, and the resin was allowed to polymerize at 60°C. The glass coverslip was removed either by heat or by treatment with hydrofluoric acid. Thin sections were stained with uranyl acetate and lead citrate and viewed in a JEOL JEM-100 CX electron microscope.

RESULTS

Purification of 14-kDa fimbrial protein and amino acid composition analysis. Upon SDS-PAGE the purified fimbrial protein preparation showed a single protein band with an approximate molecular mass of 14 kDa (Fig. 1). The amino acid composition analysis (Table 1) was similar to that reported earlier (12). However, we observed differences in the percent-

TABLE 1. Amino acid composition of fimbrial protein isolated from *S. enteritidis* AE9

Amino acid[a]	% in:	
	14-kDa fimbrial protein isolated from strain AE9	*S. enteritidis* fimbriae 14[b]
Asx	9	13
Glx	14	14
Ser	11	11
His	1	1
Gly	19	22
Arg	3	2
Thr	14	17
Pro	5	8
Ala	17	21
Tyr	2	2
Val	13	13
Ile	6	5
Leu	4	4
Phe	6	7
Lys	4	4
Trp	—[c]	1
Met	—	0
Cys	—	0
Total no. of residues/molecule	130	145
Calculated mol wt	14,400	

[a] Amino acids that differ by 1% or more are underlined.
[b] Data from reference 12.
[c] —, not detected by the procedure used.

age of some amino acids such as Ala, Pro, Thr, Arg, Gly, Ile, Phe, and Asx. The estimated molecular mass based on the amino acid composition analysis was 14.4 kDa.

Inhibition of *S. enteritidis* attachment to granulosa cells. It was possible to inhibit the attachment of *S. enteritidis* to granulosa cells by the preincubation of cells with antichicken fibronectin antibody. Preincubation of granulosa cells with a 10^{-1} dilution of the antibody resulted in approximately 70% inhibition of *S. enteritidis* attachment compared to control wells in which cells were preincubated with 0.01% bovine gamma globulin (Fig. 2). Inhibition of attachment was dependent on the dilution of antibody. At the highest dilution tested in this study (10^{-4}), nearly 21% inhibition of attachment was observed.

The effect of purified fimbrial protein (14 kDa) on the attachment of *S. enteritidis* to granulosa cells was tested by preincubation of the granulosa cells with various concentrations of the fimbrial protein. When granulosa cells were preincubated with 20 μg of fimbrial protein, the radioactivity recovered from the test wells indicated an approximate 68% inhibition of attachment (Fig. 3).

Similarly, inhibition of attachment was achieved by preincubation of bacteria with synthetic peptides known to mediate the interaction of extracellular matrix proteins with cell surfaces and macromolecules. When bacteria were incubated with 500 μg of the RGDS or GRGD peptide per ml prior to exposure to granulosa cells, a 60% inhibition of attachment was observed. In contrast, preincubation of bacteria with the GRAD peptide did not result in significant inhibition of *S. enteritidis* attachment to granulosa cells (Fig. 4). The extents of inhibition achieved with the RGDS and GRGD peptides were similar. Although the degree of inhibition caused by GRGD at 250 μg/ml appears to be lower than that of RGDS, the difference was not statistically significant. In contrast, the inactive control GRAD did not have any significant inhibitory effect on attachment (about 10 to 20% inhibition at both concentrations tested). At the maximum concentration tested in this study (500 μg/ml), both RGDS and GRGD were effective in inhibiting 60% of *S. enteritidis* AE9 attachment to granulosa cells.

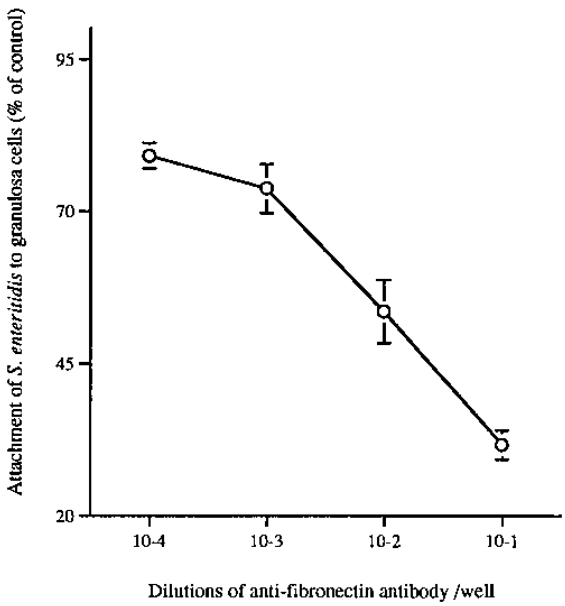

FIG. 2. Inhibition of in vitro attachment of *S. enteritidis* to chicken ovarian granulosa cells, using rabbit anti-chicken fibronectin antibody. Control wells were incubated with 0.01% rabbit gamma globulin. Error bars indicate standard error of mean (n = 9 wells, 3 experiments).

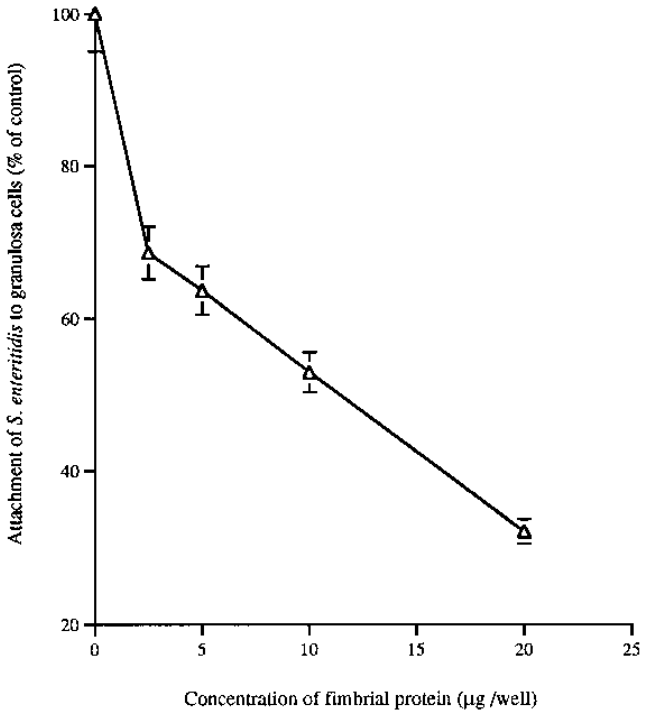

FIG. 3. Inhibition of in vitro attachment of *S. enteritidis* to chicken ovarian granulosa cells, using a 14-kDa fimbrial protein isolated from strain AE9. Control wells were incubated with 0.1% BSA. Error bars indicate standard error of mean (n = 9 wells, 3 experiments).

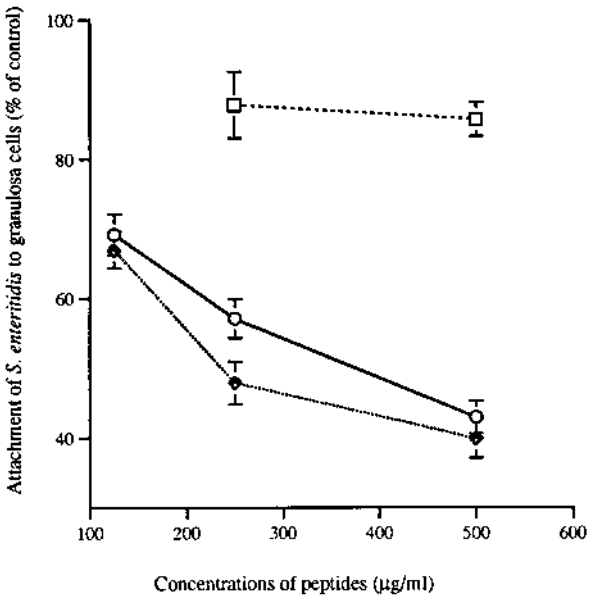

FIG. 4. Inhibition of in vitro attachment of *S. enteritidis* to chicken ovarian granulosa cells following preincubation of bacteria with the synthetic peptides Arg-Gly-Asp-Ser (RGD; circles), Gly-Arg-Gly-Asp-Asn-Pro (GRGD; diamonds) and Gly-Arg-Ala-Asp-Ser-Pro (GRAD; squares). Error bars indicate standard error of mean (n = 9 wells, 3 experiments).

Solid-phase extracellular matrix protein binding by *S. enteritidis*. The attachment of *S. enteritidis* to various extracellular matrix proteins (fibronectin, laminin, and collagen IV) was examined in 96-well tissue culture plates. The bacteria attached to all these proteins in a concentration-dependent fashion (Fig. 5). The attachment was greater in fibronectin-coated wells at all concentrations tested (Fig. 5). The rank order of attachment of *S. enteritidis* AE9 to the matrix proteins was fibronectin > laminin > collagen IV.

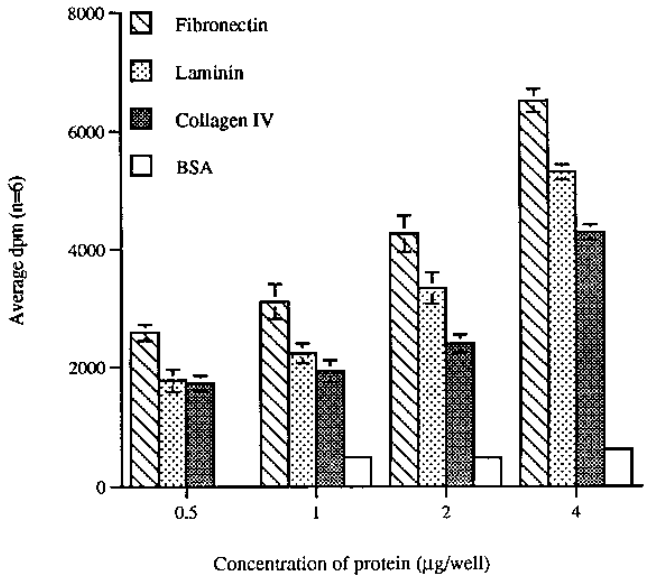

FIG. 5. Attachment of *S. enteritidis* AE9 to bovine plasma fibronectin-, mouse laminin-, and mouse collagen type IV-coated tissue culture wells. The radioactivity recovered from each test well was plotted against the concentration of protein in each well. Error bars indicate standard error of mean (n = 6 wells, 2 experiments).

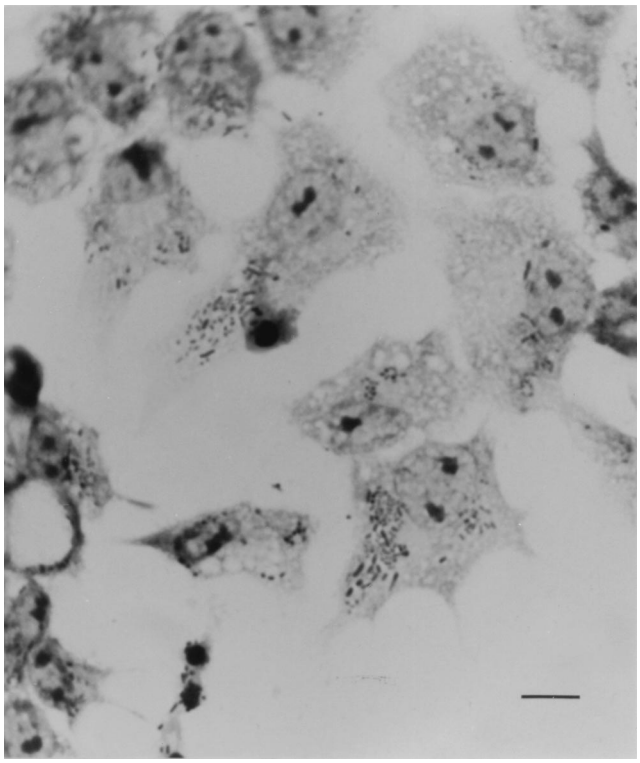

FIG. 6. Invasion of chicken ovarian granulosa cells by *S. enteritidis* AE9. Granulosa cells grown on coverslips were inoculated with *S. enteritidis* as described in Materials and Methods and stained with Giemsa stain. Bar = 10 μm.

Invasion of granulosa cells by *S. enteritidis*. Following incubation with intracellular growth medium containing gentamicin, few or no extracellular bacteria were observed under light microscopy. Intracellular, rod-shaped organisms were seen in granulosa cells (Fig. 6). It was not clear if the bacteria were enclosed in membrane-bound vacuoles. In transmission electron micrographs, the organisms can be seen as free organisms or surrounded by a membrane in the cytoplasm (Fig. 7a and c). In some micrographs, a microcolony of bacteria with dividing bacterial cells can be seen, indicating that the organism is capable of multiplying within the granulosa cells (Fig. 7b).

DISCUSSION

The phenomenon of transovarian transmission of *S. enteritidis* is an intriguing aspect of the pathogenesis of this organism in chickens. Following oral inoculation, the organism does not cause overt clinical infections in adult laying hens but leads to laying of contaminated eggs (14, 26). Invasion of intestinal walls by *S. enteritidis* leads to systemic spread of the organism (6, 14, 15, 33, 36). Previous studies indicated that following invasion of intestinal epithelial cells, *S. enteritidis* were found in macrophages in the lamina propria (32). The colonization of preovulatory follicles by blood-borne *S. enteritidis* could possibly involve interactions with the cellular components in the ovarian follicular wall (35). The follicular theca wall is a highly vascularized structure with vessels of increased permeability (18), and this anatomical feature may facilitate the transport of the organism from the blood to the developing follicle (6). Blood-borne organisms may be deposited near the basement

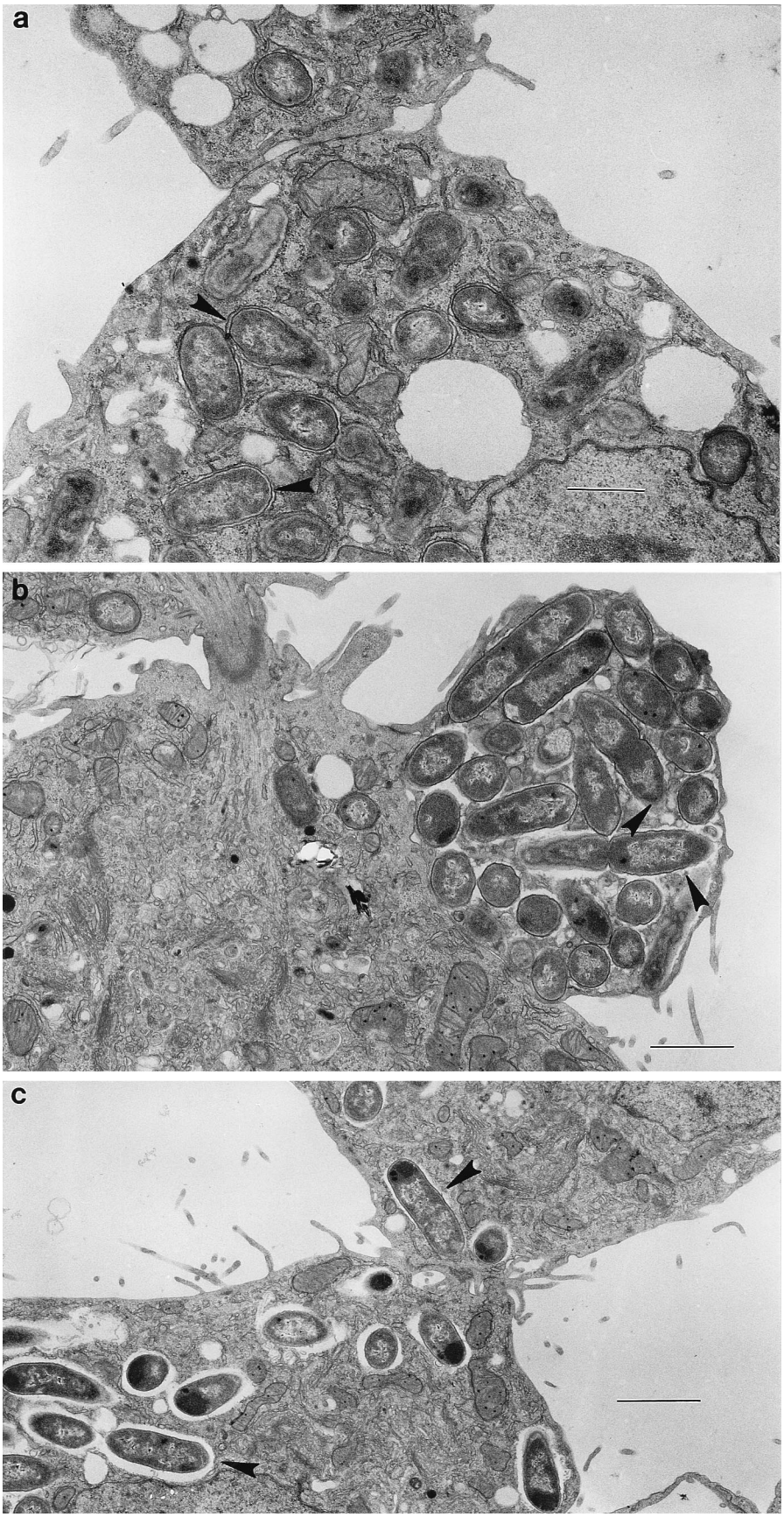

FIG. 7. Transmission electron micrographs of granulosa cell invasion by *S. enteritidis*. (a) Organisms found within the cytoplasm of the granulosa cell 5 h postinoculation. Arrowheads indicate membrane-bound bacteria in the cytoplasm (magnification, ×13,860; bar = 1 μm). (b) Microcolony of bacteria within the cytoplasm 5 h postinoculation. Arrowheads indicate bacteria in the process of division (magnification, ×10,500; bar = 1 μm). (c) Granulosa cell showing free bacteria in the cytoplasm. Arrowheads indicate the halo surrounding the bacteria (magnification, ×10,500; bar = 2 μm).

membrane itself since many of the vessels terminate near the membrane (18). From this locus, the bacteria may penetrate the basement membrane and enter the yolk after invading the granulosa cells or by migrating between the cells and traversing the perivitelline layer. It has been reported that *S. enteritidis* can attach to granulosa cells in vitro and that this may be one of the ways by which the egg becomes contaminated in vivo (35).

The in vitro attachment of *S. enteritidis* to chicken ovarian granulosa cells can be inhibited by anti-chicken fibronectin antibody, indicating that fibronectin may play a role in the attachment process. It has been shown that *S. enteritidis* binds to fibronectin (2) and that fibronectin binding may be involved in the in vitro *S. enteritidis* attachment to human intestinal epithelial cells (3). The antibody preparation used in this study recognizes fibronectin secreted by granulosa cells cultured in vitro (29). The anti-chicken fibronectin antibody may recognize several different epitopes in the fibronectin molecule that are involved in the binding of *S. enteritidis* to granulosa cells. Some of these epitopes may include cell binding domains of the fibronectin molecule since bacterial attachment was also inhibited by the RGDS and GRGD peptides (see below). Whether the cell binding domains of the fibronectin molecule are the only regions involved in the attachment process is not clear at present. Binding studies using proteolytic fragments of the fibronectin molecule are necessary to reveal the presence of other sites and their respective affinities.

To assess the significance of cell binding domain of the fibronectin molecule in the attachment process, the synthetic peptide RGDS was used to inhibit the attachment of *S. enteritidis* to granulosa cells. Previously, it has been shown that RGDS can abrogate attachment of *S. enteritidis* to granulosa cells grown on coverslips (35). In the present study, using radioactively labeled bacteria, we demonstrated that RGDS can inhibit *S. enteritidis* attachment to granulosa cells. Therefore, any extracellular matrix protein that binds macromolecules or cell surfaces through the RGD peptide sequence may be involved in this process. Two other peptides, GRGD and GRAD, were used as controls. GRGD is a strong inhibitor of fibronectin but a weak inhibitor of vitronectin binding to cell surfaces (31). GRAD is an inactive control that does not block binding of fibroblasts to fibronectin (30). When the peptide GRGD was used, the pattern of inhibition was similar to that observed for RGDS, suggesting that vitronectin may play a lesser role in the attachment process. The identity of cell surface-associated protein(s) that mediates bacteria attachment to granulosa cells under the present experimental conditions is unknown. However, fibronectin is a good candidate because the bacteria attached to fibronectin to a greater extent than laminin and collagen IV in solid-phase extracellular matrix protein binding assays. Moreover, anti-fibronectin antibody suppressed *S. enteritidis* interaction with granulosa cells. The other factors that contribute to the attachment of *S. enteritidis* to granulosa cells remain to be determined. It has been reported that attachment of several strains of *S. enteritidis* to human intestinal epithelial cells may involve binding of fibronectin (3). Additionally, it was reported that some strains bound the 29-kDa amino-terminal fragment of fibronectin whereas other strains did not. However, the latter class bound the intact fibronectin molecule, indicating that sites other than the ones at the amino-terminal domain may also be involved in *S. enteritidis* interaction with fibronectin (3). This notion is also borne out by our study in that the cell binding domain alone cannot account for the entire attachment activity.

Chicken ovarian granulosa cells have been shown to deposit fibronectin in vitro, and this deposition can be modulated by hormones (29). The expression of fibronectin seems to vary between different developmental stages of the follicles, with maximal expression appearing at the F1 follicle (most mature) stage. It is not clear how this may affect the in vivo interactions of *S. enteritidis* with granulosa cells. However, no differences were observed among the three most developed follicular structures (F1, F2, and F3) when they were used in *S. enteritidis* attachment assays (35).

S. enteritidis strains have been shown to express several types of fimbriae. Some fimbriae are involved in the agglutination of erythrocytes from various animal species (11) and adherence to mouse intestinal cells (1). In the present study, it was possible to inhibit in vitro attachment of *S. enteritidis* to granulosa cells by preincubating the cells with purified fimbrial preparation, suggesting a major role for this fimbrial protein in the attachment process. Although the 14-kDa fimbrial protein may be involved in the attachment of *S. enteritidis* AE9 to granulosa cells, the roles of other fimbrial types and their relative contributions to the attachment process remain to be examined.

The fimbrial structure(s) on *S. enteritidis* may provide binding sites to a variety of extracellular matrix proteins (2, 3, 7, 23). In the present study, strain AE9 interacted with fibronectin, laminin, and collagen IV. It remains to be seen whether this interaction involves 14-kDa fimbrial protein alone or other fimbrial structures on the bacterial surface. Binding to laminin and collagen IV may be relevant because in vivo access to granulosa cells would involve binding to the basement membrane, which is rich in collagen IV and laminin (37).

It was demonstrated in this study that *S. enteritidis* invades the granulosa cells. This finding implies that ovarian granulosa cells may be one type of target cell involved in the transovarian transmission of the organism. In transmission electron micrographs, it was possible to see cytoplasmic bacteria surrounded by a membrane and in some instances as free bacteria in the cytoplasm (Fig. 7a and c). The presence of a membrane suggests that the organisms may have been endocytosed by invagination of the granulosa cellular membrane. The nonvacuolated bacteria found within the granulosa cells are often surrounded by a halo without a well-defined membrane (Fig. 7b). The seeming occurrence of free organisms in the cytoplasm may not be real. It is possible that the membrane-bound vacuoles were not well preserved in these specimens during fixation. Alternatively, they may represent organisms that actively invaded the granulosa cells. It is speculated that the halo surrounding the organisms may represent areas within the cytoplasm lysed by bacterial toxins or enzymes. The cytopathic effect of *S. enteritidis* on granulosa cells and its significance in vivo requires further examination. In the present study, some of the bacterial cells in membrane-surrounded vacuoles were dividing, suggesting that *S. enteritidis* is capable of multiplying in granulosa cells (Fig. 7b) as has been described for other cell types. It is possible that dividing organisms were present in the culture medium before addition to granulosa cells. Additional time course studies are required to determine if the organisms can multiply in granulosa cells. It would be interesting to determine whether invasion followed by multiplication is a prerequisite for transovarian transmission of *S. enteritidis*.

In summary, results presented in this study indicate that *S. enteritidis* interacts with granulosa cells in a specific manner. This interaction may be largely mediated by surface structures on the bacterial and target cells. *S. enteritidis* may also invade and multiply in ovarian granulosa cells. Since the granulosa cell layer of the preovulatory follicle is located close to the egg yolk mass, it is conceivable that the contamination of egg yolk prior to oviposition explains the mechanism of transovarian transmission of *S. enteritidis*. Furthermore, a few *S. enteritidis*-con-

taining granulosa cells may be dislodged from the basement membrane and released together with the yolk mass during ovulation. These processes may lead to the production of *Salmonella*-contaminated eggs, which is the subject of significant public health concern.

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