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Lactoferrin-Binding Proteins in *Shigella flexneri*

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The ability of *Shigella flexneri* to interact with lactoferrin (Lf) was examined with a ^{125}I-labeled protein-binding assay. The percent binding of human lactoferrin (HLf) and bovine lactoferrin (BLf) to 45 *S. flexneri* strains was 19 ± 3 and 21 ± 3 (mean ± standard error of the mean), respectively. ^{125}I-labeled HLf and BLf binding to strain M90T reached an equilibrium within 2 h. Unlabeled HLf and BLf displaced the ^{125}I-HLf–bacteria interaction in a dose-dependent manner. The Lf-bacterium complex was uncoupled by KSCN or urea, but not by NaCl. The interaction was specific, and ~4,800 HLf binding sites (affinity constant [K_a], 690 nM) or ~5,700 BLf binding sites (K_a, 104 nM) per cell were estimated in strain M90T by a Scatchard plot analysis. The native cell envelope (CE) and outer membrane (OM) did not reveal Lf-binding components in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, after being boiled, the CE and OM preparations showed three distinct horseradish peroxidase-Lf reactive bands of about 39, 22, and 16 kDa. The 39-kDa component was also reactive to a monoclonal antibody specific for porin (PoI) proteins of members of the family *Enterobacteriaceae*. The Lf-binding protein pattern was similar with BLf or HLf, for Crb$^+$ and Crb$^-$ strains. The protein-Lf complex was dissociable by KSCN or urea and was stable after treatment with NaCl. Variation (loss) in the O chain of lipopolysaccharide (LPS) markedly enhanced the Lf-binding capacity in the isogenic rough strain SFL1070-15 compared with its smooth parent strain, SFL1070. These data establish that Lf binds to specific components in the bacterial OM; the heat-modifiable, anti-PoI-reactive, and LPS-associated properties suggested that the Lf-binding proteins are porins in *S. flexneri*.

Shigella flexneri is the most common etiological agent of bacillary dysentery, especially that contracted by children in developing countries (6, 37). Bacterial adhesion to colonic epithelia, cell invasion, and multiplication in host tissues are necessary for the onset of shigellosis (19, 27, 45). The formation of ulcerative lesions, as well as the infiltration of erythrocytes and inflammatory cells, mainly polymorphonuclear leukocytes, into the bowel, is an early event in the pathogenesis (20, 27). It has been suggested that intense inflammatory responses prevent further systemic dissemination of *S. flexneri* (37). Various antimicrobial, acute-phase proteins secreted by the activated polymorphonuclear leukocytes may play an important role in the inflammatory host defense (7, 55). However, our knowledge of the interaction of acute-phase proteins with *S. flexneri* is limited.

Lactoferrin (Lf) is an antimicrobial acute-phase protein secreted by polymorphonuclear leukocytes as well as by exocrine glands (3, 7, 8, 33–35) and is found in milk and at the mucosal surface. The mechanism of Lf-mediated antimicrobial action has mainly been attributed to its iron deprivation capacity with bacteria (47). The antimicrobial activity seems to be enhanced when Lf adsorbs to bacteria (2, 12, 16). Furthermore, specific receptors for Lf were reported in the mammalian intestinal brush border (11, 14) and certain peptides from Lf were shown to inhibit the adherence of *S. flexneri* to enterocytes (24).

On the other hand, the ability of bacteria to compete for iron within the host is one of the factors that influence pathogenicity (49, 56). Under iron stress, *S. flexneri* may produce one or more siderophores in vitro, for acquiring the Fe^{3+} ion (48). *S. flexneri* may also utilize various host iron compounds, including Lf, transferrin, hemin, and hematin, in vitro (29). Furthermore, a 101-kDa heme-binding protein in *S. flexneri* has been identified (53). In pathogens such as those belonging to the genus *Neisseria*, Lf-binding proteins were also identified and attributed to iron acquisition (39, 52). Thus, Lf-bacterium interaction may be relevant in bacterial virulence and host defense. However, the interaction of Lf with *S. flexneri* has not been reported.

In this communication, we give evidence for the specific binding of Lf to *S. flexneri*. The nature of the Lf-bacterium interaction has been characterized, and the outer membrane (OM) proteins involved in Lf binding in *S. flexneri* M90T were identified.

MATERIALS AND METHODS

Bacterial strains. A total of 45 *S. flexneri* strains comprising 20 fecal isolates from gastroenteritis patients and 23 type strains (kindly supplied by É. Czirok and H. Milch, National Institute of Hygiene, Budapest, Hungary) were tested. Strains M90T (Crb$^+$) and M90T55 (Crb$^-$) were provided by T. Pál, Institute of Microbiology, University Medical School, Pécs, Hungary. In addition, a wild-type strain, SFL1070, containing smooth lipopolysaccharide (LPS) (Δ*aroD*, serotype 2a) and its isogenic mutant, SFL1070-15, with rough LPS were provided by A. Lindberg, Department of Clinical Bacteriology, Karolinska Institute, Stockholm, Sweden. Bacterial strains were grown in antibiotic (Penassay) medium 3 (Difco Laboratories, Detroit, Mich.) with continuous mechanical shaking (100 rpm) at 37°C for 24 h. Cells were harvested, washed, and resuspended in phosphate-buffered saline (PBS), pH 7.2. The cell density was adjusted photometrically (at 600 nm) to 10^{10} bacteria per ml for binding studies.

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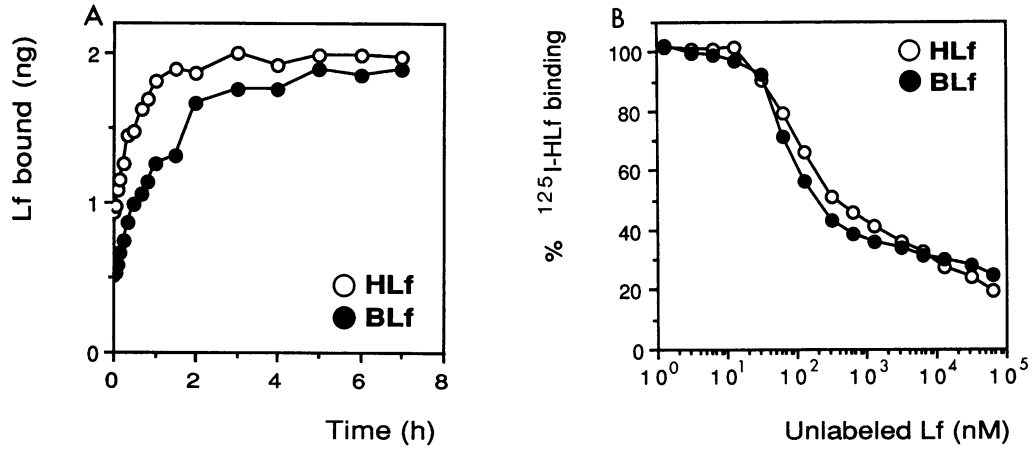

FIG. 1. (A) Kinetics of ^{125}I-HLf and ^{125}I-BLf binding equilibrium with strain M90T. Bacteria (10^9 cells in 0.1 ml) were incubated with ~8 ng of ^{125}I-Lf (~30 kcpm) in 0.1 ml of PBS at 37°C. At different time points (as indicated), the reaction was terminated by adding 2 ml of ice-cold PBS containing 0.1% Tween 20. The supernatant was aspirated after centrifugation (5,000 × *g*), and the radioactivity bound to the bacterial pellet was measured. The values shown represent binding after deducting the background radioactivity measurements. (B) Displacement of ^{125}I-labeled HLf binding by increasing amounts of unlabeled HLf or BLf. Approximately 10^9 cells (in 0.1 ml of PBS) of strain M90T were mixed with ~8 ng of ^{125}I-HLf (in 0.1-ml volumes) and incubated at 37°C for 2 h. Increasing amounts (1 to 10^5 nM) of unlabeled HLf or BLf (in 0.1-ml volumes, diluted in PBS) were added to the HLf-bacterium complex (final volume, 0.3 ml) and further incubated for an additional hour. The ^{125}I-labeled protein-binding measurements were made as described in the text, and the homologous (^{125}I-HLf versus unlabeled HLf, r = 0.98) and heterologous (^{125}I-HLf versus unlabeled BLf, r = 0.97) displacement curves were plotted.

Chemicals. Human lactoferrin (HLf) (lot 63541) purified from milk was purchased from the U.S. Biochemicals Corp., Cleveland, Ohio. Bovine lactoferrin (BLf) purified from milk whey was kindly provided by H. Burling, Swedish Dairies Association, Malmö, Sweden. Both Lf preparations were homogeneous in ion-exchange (Mono-Q column; Pharmacia, Uppsala, Sweden) and in molecular-sieve (TSK G4000 SW; LKB Produkter AB, Bromma, Sweden) high-performance liquid chromatography. Apo-Lf was prepared by removing iron from the protein by exhaustive dialysis against 0.1 M citrate (1). Transferrin (apo form, lot 67F.9457; iron-saturated form, lot 67F.9458), hemin (bovine, type I, lot 104F.0090), mucin type III (porcine stomach, lot 49F.3876), sodium deoxycholate, *N*-acetylglucosamine (lot 87F.0329), *N*-acetylgalactosamine (lot 77F.5053), *N*-acetylneuraminic acid (sialic acid type VIII from sheep submaxillary glands, lot 28F.1046), *N*-acetylmuramic acid (lot 18F.1016), Congo red (lot 118F-3494), and peroxidase type VIA from horseradish (HRPO) (EC 1.11.1.7, lot 69F.9525) were purchased from Sigma Chemical Co., St. Louis, Mo. All chemicals used for the preparation of buffer solutions were of analytical grade.

Congo red binding (Crb) and HeLa cell invasion assay. Congo red (0.01%, wt/vol) was incorporated into Trypticase soy broth and solidified with 1.5% agar. Crb$^+$ strains bound the dye and demonstrated red colonies. The invasive capacity of the strains was examined with HeLa cell monolayers as previously described (46).

^{125}I-Lf binding assay. HLf and BLf were labeled with Na^{125}I (specific activity, 629 GBq/mg; DuPont Scandinavia AB, Stockholm, Sweden) by using Iodobeads (Pierce Chemical Co., Rockford, Ill.) (32). Binding assays were performed as described previously by Naidu et al. (40, 41). Briefly, 10^9 bacteria (in 0.1 ml of PBS) were mixed with 0.1 ml of PBS containing ~8 ng of ^{125}I-Lf (specific activity, 0.16 MBq/μg). After incubation for 2 h at 37°C (according to binding equilibrium kinetics [Fig. 1A]), the binding reaction was terminated by adding 2 ml of ice-cold PBS (containing 0.1% Tween 20). The suspension was centrifuged at 5,300 × *g* for 15 min, and the supernatant was aspirated. Radioactivity bound to the bacterial pellet was measured in a gamma counter (Clinigamma 1272; LKB Wallac, Turku, Finland). The background radioactivity (from incubation mixtures without bacteria) was deducted, and the binding was expressed in percentages calculated from the total labeled ligand added to the bacteria. *Escherichia coli* E34663 and HH45 served as positive (60%) and negative (5%) binding controls, respectively.

A moderately Lf-binding *S. flexneri* laboratory strain, M90T, and a clinical isolate, SH10, were selected for characterization studies. Both strains showed similar binding characteristics; therefore, the data obtained with strain M90T are presented.

Dissociation of Lf-bacterium complex. Binding of ^{125}I-Lf to bacteria was performed as described above, and the following reagents of various molarities, i.e., 1 ml each of sodium chloride (1 to 5 M), potassium thiocyanate (1 to 5 M), and urea (1 to 8 M), were added to the ligand-bacterium complex. Reaction mixtures containing 1 ml of 20 mM potassium phosphate buffer, pH 7.2, served as the control (taken as 100% binding). After incubation at 37°C for 1 h, the mixture was centrifuged (5,300 × *g* for 15 min), the supernatant was aspirated, and the radioactivity bound to the bacterial pellet was measured.

Isolation of bacterial CE and OM. Strains M90T (Crb$^+$) and M90T55 (Crb$^-$) were grown in 400 ml of Penassay medium, harvested (100 × *g* for 15 min), and washed twice with 10 mM Tris HCl, pH 7.4, and the cell density was adjusted photometrically to ~4 × 10^{10} bacteria per ml in the same buffer containing 10 mM $MgCl_2$. Cells were sonicated (200 W, three times for 2 min each) on ice, and the sonicate was centrifuged (3,000 × *g* at 4°C for 15 min). The resulting supernatant was ultracentrifuged (Beckman L7; 100,000 × *g*, at 4°C for 1 h), and a pellet called the "cell envelope" (CE) was obtained. The CE preparation was further treated with 2% (vol/vol) Triton X-100 (Sigma) in 10 mM Tris HCl

containing 10 mM $MgCl_2$ and was kept at 37°C for 1 h with end-to-end rotation. After an additional ultracentrifugation step as described above, a pellet (designated the OM pellet) was obtained. Both CE and OM preparations were dissolved in 0.06 M Tris HCl, pH 6.8, containing 2% (wt/vol) sodium dodecyl sulfate (SDS) and 10% (vol/vol) glycerol and were stored at −20°C.

SDS-PAGE and probing of the CE and OM with HRPO-labeled Lf. The CE and OM preparations were mixed with a sample buffer (0.06 M Tris HCl, pH 6.8; containing 5% β-mercaptoethanol, 2% SDS, and 10% glycerol), and also other CE and OM preparations were boiled in the above buffer at 100°C for 5 min. Both boiled and unboiled samples were tested by performing SDS-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (28) in a Miniprotean II apparatus (Bio-Rad, Richmond, Calif.). The gel consisted of 12% acrylamide and 0.31% bisacrylamide, and the electrophoresis was run at 60 V for ~2 h.

After SDS-PAGE, proteins were electrophoretically transferred from the gel to a nitrocellulose membrane (Sartorius, Göttingen, Germany) at 200 mA for 2 to 3 h, by using semidry blot equipment (Novablot 2117-250; Pharmacia LKB). After being blocked with Tween 20 (1%, vol/vol) in Tris-buffered saline, pH 7.4 (TBS), for 90 min, the membrane was washed three times with TBS. HLf and BLf were coupled to HRPO by a periodate method (43). HLf- or BLf-HRPO (dilution, 1:350 in TBS) was added to the membrane and incubated at room temperature for 2 h. After the membrane was washed three times with TBS containing 0.05% Tween 20, a color reaction was developed with diaminobenzidine (0.25 mg/ml; Sigma) chromophore dissolved in 0.1 M sodium acetate buffer, pH 5.0, containing hydrogen peroxide (0.003%, vol/vol), and the reaction was terminated by adding 5% sodium pyrosulfite (1%, wt/vol). Preincubation of blots with unlabeled HLf abolished the HLf-HRPO interaction and excluded the possibility of direct HRPO binding to bacterial components.

Immunoblotting with an anti-porin MAb probe. Monoclonal antibodies (MAb) against a conserved porin protein domain (PoI) common to 10 different genera of the family *Enterobacteriaceae*, including *S. flexneri*, previously described by Henriksen and Mæland (22) were used. The boiled and unboiled CE preparations were run on SDS-PAGE gels, and the proteins in the gel were transblotted to a nitrocellulose membrane. Free sites on the membrane were blocked with TBS (containing 1% ovalbumin and 1% Tween 20) for 30 min. Anti-PoI antibody (MAb F9-16) was mixed with the blocking solution at a working dilution of 1:5,000, and the solution was incubated at room temperature for 2 h with gentle shaking. After the membrane was washed thoroughly with TBS, it was incubated with HRPO-labeled rabbit antibodies to mouse immunoglobulins (lot 059; Dakopatts A/S, Glostrup, Denmark) at room temperature for 1 h with gentle shaking. After a final washing step, the membrane was developed with diaminobenzidine chromophore. Strips incubated with only labeled second antibody served as controls.

LPS analysis by SDS-PAGE. Strain SFL1070 and its mutant, SFL1070-15, were examined for LPS patterns as previously described (18). The sarcosyl-extracted OM preparations from the bacteria were boiled for 10 min and digested with proteinase K (30 to 60 U/ml; Sigma) at 37°C for 2 h. The preparations were run on SDS-PAGE gels as described above, and the gels were stained with a silver staining kit (Bio-Rad; lot 35709) according to the manufacturer's instructions.

TABLE 1. ^{125}I-labeled HLf and BLf binding to *S. flexneri* strains[a]

Strain	Serotype or subsp.	% Binding (mean ± SEM) to:	
		^{125}I-HLf	^{125}I-BLf
NCTC 9950	Type 1a	13 ± 3	19 ± 5
63-125-700	Type 1a	16 ± 2	19 ± 2
DRL41/53	Type 1b	10 ± 1	14 ± 3
OKI2/68	Type 1b	11 ± 1	11 ± 1
26D	Type 2a	16 ± 1	20 ± 1
Sc592	Type 2a	13 ± 1	17 ± 1
Sc661	Type 2b	14 ± 3	19 ± 3
DRL5/63	Type 3a	14 ± 1	20 ± 2
OKI264	Type 3a	16 ± 1	21 ± 2
1308/68/2	Type 3b	16 ± 1	18 ± 1
PHLS11D	Type 4a	11 ± 2	13 ± 2
Carpenter IV	Type 4a	16 ± 2	20 ± 1
NCTC 8522	Type 4b	10 ± 1	12 ± 2
OKI840	Type 4b	14 ± 1	15 ± 1
M90T	Type 5	17 ± 1	19 ± 2
M213 x$^-$	Type 5	14 ± 2	16 ± 2
OKI273	Type 5	16 ± 1	20 ± 1
67-104-1	Type6	13 ± 1	10 ± 1
NCTC 9729	Type 6	12 ± 1	15 ± 1
Newcastle	6 subsp. newcastle	16 ± 2	22 ± 2
DRL531/61	Subsp. *X*	12 ± 1	14 ± 2
OKI222	Supsp. *X*	14 ± 2	18 ± 2
Ledingham	Subsp. *Y*	12 ± 1	12 ± 1
NCTC 9730	Subsp. *Y*	12 ± 1	15 ± 1

[a] Bacteria grown in Penassay broth were tested for Lf interaction in a ^{125}I-labeled protein-binding assay as described in the text. Values represent the percent Lf bound to 10^9 bacteria incubated with ~8 ng of ^{125}I-protein for 2 h (SEM based on four experiments).

RESULTS

The magnitude of ^{125}I-labeled HLf and BLf binding (expressed as the percent Lf bound from 8 ng of labeled ligand added) to *S. flexneri* type strains (n = 24) is shown in Table 1. The type strains and an additional 20 clinical isolates demonstrated mean percent (mean ± standard error of the mean [SEM]) binding of 19 ± 3 (minimum, 10; maximum, 33) and 21 ± 3 (minimum, 10; maximum, 36) for HLf and BLf, respectively.

The nature of the Lf-*S. flexneri* interaction was characterized in strain M90T. Strain M90T (10^9 bacteria) demonstrated a binding equilibrium within 2 at ~8 ng of ^{125}I-labeled HLf or BLf per ml (Fig. 1A). Of the various unlabeled proteins and carbohydrates tested, only Lf from bovine and human species strongly inhibited (~90%) the ^{125}I-labeled Lf binding to strain M90T, both in homologous and heterologous combinations (Table 2). The iron-saturated Lf preparations were slightly more inhibitory than the apo forms. Iron-binding proteins (i.e., transferrin and hemin) and other commonly occurring substances of the gastrointestinal tract, such as mucin, deoxycholate, and glycoconjugates (i.e., *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, and *N*-acetylmuramic acid), caused less than 30% inhibition of the Lf binding. The displacement of ^{125}I-HLf binding to bacteria by homologous (HLf) or heterologous (BLf) unlabeled Lfs was dose dependent and required ~4 μg of protein per ml to elicit a 50% effect (Fig. 1B). The stability of the ^{125}I-HLf–bacterium complex was examined in a whole-cell binding assay with different chemical agents from 0 to 5 M (Fig. 2). The binding was stable in the presence of 5 M NaCl. However, KSCN and urea caused

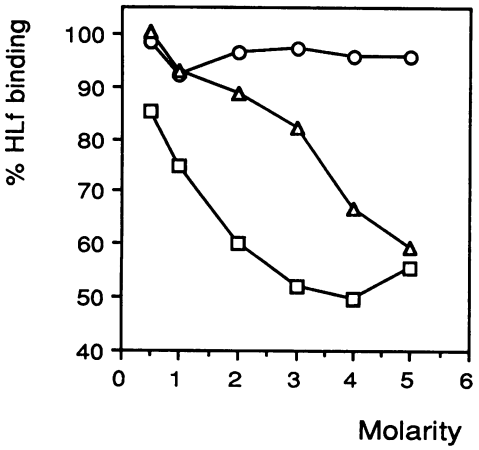

FIG. 2. Stability of ^{125}I-HLf–bacterium interaction in strain M90T. The ^{125}I-HLf binding to bacteria was performed at 37°C for 2 h, as described in the text. The reaction was terminated by adding ice-cold PBS-Tween; after centrifugation, the radioactivity bound to the bacterial pellet was measured. Various molarities (in 1-ml volumes) of NaCl (○), KSCN (□), or urea (▽) were added to tubes containing the ligand-bacterium complex; control tubes received 1 ml of 20 mM phosphate buffer, pH 7.2. The mixtures were allowed to stand for an additional 1 h at 37°C and were then centrifuged (5,300 × *g* for 15 min); the supernatant was aspirated, and the radioactivity retained with the bacterial pellet was measured. Binding was expressed in percentages relative to that of the controls (100%).

a concentration-dependent dissociation of the HLf-bacterium complex.

The concentration-dependent saturability of ^{125}I-HLf and ^{125}I-BLf binding to strain M90T was estimated (Fig. 3). The magnitude of nonspecific binding was higher for the bacterial interaction with HLf than with BLf. In a limited time of 2 h, approximately 5 μg of HLf or 7.5 μg of BLf could saturate Lf binding to ~10^9 bacterial cells. The specific binding values obtained from Fig. 3 were analyzed in a Scatchard plot (50) (Fig. 4). Thus, 4,800 HLf-bacterium binding sites, with an affinity constant (K_a) of 690 nM, and 5,600 BLf-bacterium binding sites (K_a, 104 nM) were estimated for strain M90T.

TABLE 2. ^{125}I-labeled HLf and BLf binding to strain M90T in the presence of various unlabeled proteins and carbohydrates[a]

Substance (0.1 mg/ml)	% Binding (mean ± SEM)	
	^{125}I-HLf	^{125}I-BLf
Control (PBS)	100	100
HLf (apo)	13 ± 1	13 ± 1
HLf (iron saturated)	10 ± 2	12 ± 1
BLf (apo)	11 ± 1	9 ± 1
BLf (iron saturated)	9 ± 1	8 ± 1
Transferrin (apo)	77 ± 1	75 ± 3
Transferrin (iron saturated)	78 ± 1	77 ± 3
Hemin	70 ± 2	71 ± 4
Mucin type III	70 ± 2	75 ± 2
Sodium deoxycholate	95 ± 2	93 ± 1
N-acetylglucosamine	80 ± 6	82 ± 4
N-acetylgalactosamine	82 ± 8	78 ± 5
N-acetylneuraminic acid	81 ± 5	73 ± 1
N-acetylmuramic acid	77 ± 6	76 ± 2

[a] Bacteria (10^9 cells in 0.1 ml) were incubated with unlabeled protein or carbohydrate (10 μg in 0.1 ml) at 37°C for 1 h. A volume of 0.1 ml of ^{125}I-labeled HLf or BLf (~8 ng, corresponding to 30 kcpm) was added to the above mixture and incubated at 37°C for 2 h with gentle shaking. The binding experiment was performed as described in the text. The percent binding was calculated from ^{125}I-Lf binding to cells in PBS (control) (SEM based on four experiments).

The presence of Lf-binding components in the CE and OM of strains M90T (Crb$^+$) and M90T55 (Crb$^-$) was examined by using HRPO-labeled Lf by Western blot (ligand blot) analyses (Fig. 5). Unheated CE and OM preparations did not show any Lf-binding components. However, after boiling, the same preparations revealed three distinct HRPO-Lf reactive bands of approximately 39, 22, and 16 kDa. Of these three heat-modifiable CE components, only the 39-kDa protein was weakly reactive against anti-PoI antibody. The pattern of Lf-binding proteins was similar for BLf and HLf in Crb$^+$ and Crb$^-$ strains. Furthermore, the stability of the receptor-HLf complex in the presence of NaCl, KSCN, and urea was tested by Western blot (Fig. 6). The HRPO-HLf-receptor complex readily dissociated with increasing molarities of KSCN or urea, but not with NaCl, which is similar to the Lf-whole cell dissociation results shown in Fig. 4.

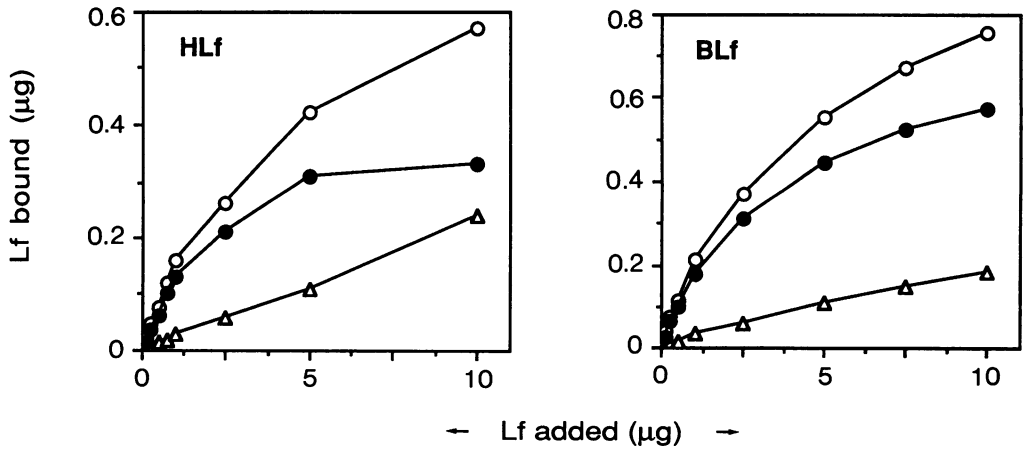

FIG. 3. Saturability of specific binding of ^{125}I-labeled HLf and -BLf to strain M90T. Increasing amounts of ^{125}I-labeled Lf (range, 0.1 to 10 μg) in 0.1-ml volumes were added to bacteria (8 × 10^8 cells per 0.1 ml), and the mixture was kept at 37°C for 2 h. Binding was performed in the absence (○ [total binding]) or presence (▽ [nonspecific binding]) of 50-fold excess unlabeled ligand. Specific binding (●) was calculated by subtracting the nonspecific Lf uptake values from total binding values.

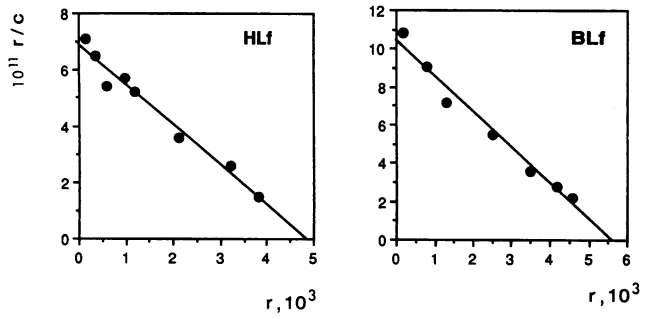

FIG. 4. Scatchard plot analysis ($r/c = nK_a - rK_a$) of ^{125}I-Lf binding to *S. flexneri* M90T. The molecules of Lf bound to one bacterial cell (r) and the molecules of Lf free in the medium (c) were calculated by assuming molecular masses of 82,400 and 92,100 daltons for HLf and BLf, respectively (38, 57). The intercept on the x axis represents the number of receptors per cell (n). The slopes of the lines represent the effective K_a (expressed as the nanomolar concentration).

The ^{125}I-HLf-binding capacity of the parent strain SFL1070 was 15 ± 1% (mean ± SEM), and the corresponding value for the isogenic derivative SFL1070-15 with rough LPS was 42 ± 2% (mean ± SEM) (about threefold higher binding). The boiled OM of the rough strain showed an incomplete O chain in the LPS structure compared with the smooth LPS of the parent strain; however, both strains demonstrated similar Lf-binding protein patterns (Fig. 7).

DISCUSSION

Breast-feeding has been suggested to decrease the frequency of symptomatic shigellosis and also the severity of the illness (10, 36). This protection has been attributed to various components in milk such as immunoglobulins, nonspecific cell receptor analogs, and Lf (8, 9). Lf has been shown to elicit bacteriostatic and also bactericidal effects (2, 34, 47). An enhanced antimicrobial activity could be achieved when Lf adsorbs to the bacteria (12, 16). Recently, we have described specific receptors for Lf in certain bacteria causing intestinal infections, i.e., *E. coli* and *Aeromonas hydrophila* (25, 42). The present study also demonstrates the binding of HLf and BLf to specific outer membrane proteins (OMPs) in *S. flexneri*.

The binding of HLf and BLf to strain M90T reached an equilibrium within a definite time and implied that there are a limited number of interaction sites on the bacterium. Binding inhibition studies indicated that strain M90T interacts with apo- and iron-saturated forms of Lf from human and bovine species. Extraintestinal pathogens, i.e., *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae*, seem to recognize only HLf but not BLf (30, 51, 52). Furthermore, various compounds common in the gastrointestinal tract caused little or no effect on Lf binding to bacteria. The ^{125}I-HLf binding displacement studies suggested that the bacterium recognized both isotope-labeled and unlabeled forms of ligands, preferably the latter form. The binding was reversible, hence the interaction seems to be of a low affinity. The binding characteristics of *S. flexneri* seem to be similar to those reported for *E. coli* and *A. hydrophila* (25, 42).

We have recently demonstrated that Lf recognizes porins OmpC and OmpF in *E. coli* (17). A 40-kDa Lf-binding protein in *A. hydrophila* (25) also seems to be a previously described pore-forming protein (13). Lf also binds to porins in a *Salmonella* sp. and potentiates the action of antibiotics by altering the permeability of the bacterial OM (26). The boiled preparations of CE and OM of *S. flexneri* M90T revealed three Lf-binding proteins of 39, 22, and 16 kDa in an SDS-PAGE and Western blot analysis. The Lf-binding protein pattern was similar for HLf and BLf among Crb$^+$ and Crb$^-$ variants of strain M90T. However, the corresponding protein bands were absent in the native (unboiled) CE and OM preparations. This heat-modifiable property is a characteristic feature for porins (the pore-forming OMPs) (4, 31). Henriksen and Mæland (22) have described an MAb that could specifically recognize a conserved domain in the PoI protein in a majority of bacteria belonging to the family *Enterobacteriaceae*. The 39-kDa heat-modifiable, Lf-binding

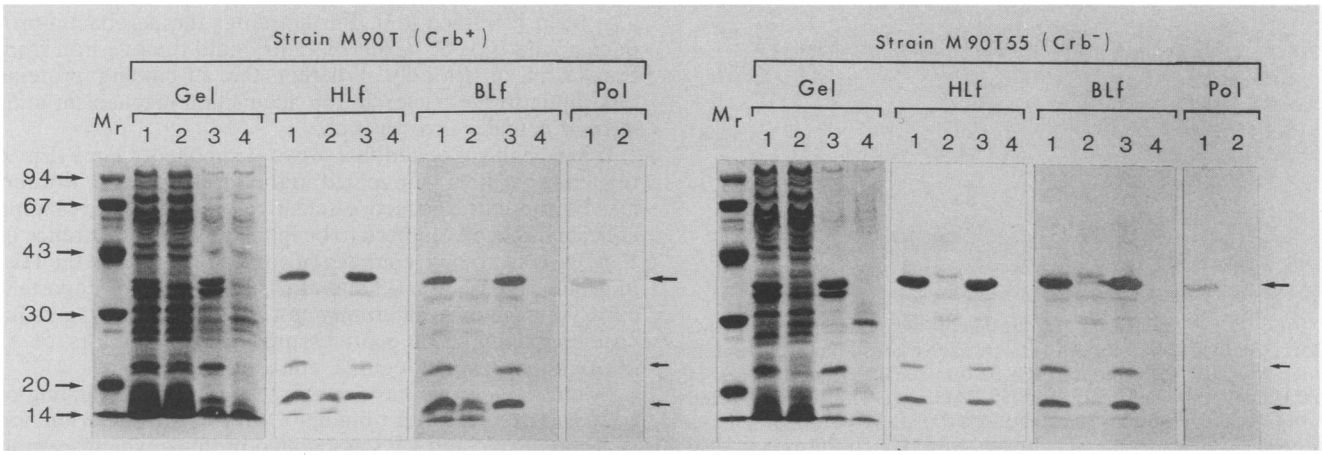

FIG. 5. Demonstration of Lf-binding proteins and porins in Crb$^+$ and Crb$^-$ strains of *S. flexneri* by Western blotting after SDS-PAGE. The CE and OM proteins of Crb$^+$ M90T and its Crb$^-$ variant, M90T55, were analyzed as native and boiled preparations by SDS-PAGE (12% acrylamide, 0.31% bisacrylamide), and the electrophoresis was run at 60 V for ~2 h. Proteins from unstained gels were transblotted to a nitrocellulose membrane, and the free sites on the membrane were blocked. The membranes were probed with HRPO-labeled HLf, HRPO-labeled BLf, or PoI (anti-porin MAb F9-16) as described in the text. Lanes 1, CE boiled at 100°C for 5 min; lanes 2, native CE; lanes 3, OM boiled at 100°C for 5 min; lanes 4, native OM. The gel was stained with Coomassie brilliant blue R. The molecular mass standards (kilodaltons) are indicated by arrows to the left, and the HRPO-Lf-reactive bands are indicated by arrows to the right of each gel.

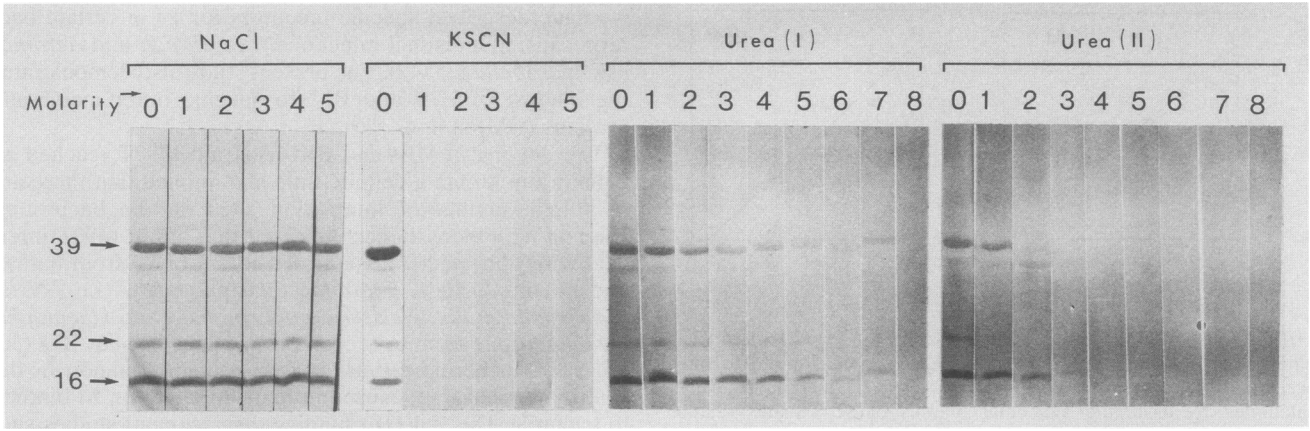

FIG. 6. Effect of various chemical agents on the bacterial OMP-Lf complex. The heat-treated OMPs of strain M90T were run on SDS-PAGE gels and transblotted to a nitrocellulose membrane, and free sites on the membrane were blocked. Each membrane strip was incubated with HLf-HRPO conjugate for 2 h and thoroughly washed with TBS-Tween. The strips were further incubated with various molarities (in 2-ml volumes) of NaCl, KSCN, or urea [Urea(I)] for 1 h. After a thorough wash (three times) with TBS-Tween, the strips were developed with diaminobenzidine as described in the text. An additional set of strips was treated with urea prior to the binding reaction with HLf-HRPO [Urea(II)]. Arrows to the left indicate the molecular masses (kilodaltons) of the HLf-HRPO-reactive bands.

component identified in *S. flexneri* was reactive against this immunoprobe. Porins in their native trimeric form associate with LPS and dissociate into monomers by boiling in SDS (15). Some of these porins perform key functions such as selective uptake of various nutrients and serving as receptors for bacteriophages and colicins (4, 31, 44). The O chain of LPS associated with porins may shield the receptor function (54). In this study, we have also shown a similar LPS O chain-mediated masking of Lf binding in *S. flexneri* by using a smooth strain and its isogenic rough derivative. We have also demonstrated in a previous study that the smooth LPS associated with native porins blocks *E. coli* from Lf interaction (18). Thus, the Lf-binding components in *S. flexneri* also appear to be porins. Though porins occur at a copy number of 10^5 per bacterium (4, 31), an estimated 20-fold less binding sites per cell in strain M90T in Scatchard plot analyses may be due to the availability of a limited number of porins for Lf interaction. The Scatchard plot was linear and implied a single class of receptors; the 22- and 16-kDa Lf-binding components detected in the Western blot are possibly cleaved products of the porin.

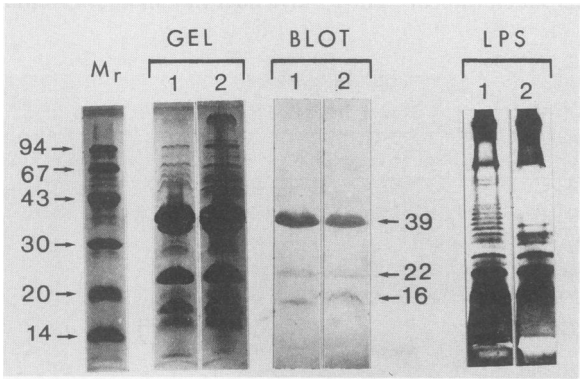

FIG. 7. LPS and Lf-binding protein profile of the *S. flexneri* OM. OMs isolated from a smooth LPS strain, SFL1070 (lanes 1), and its isogenic rough LPS mutant, SFL1070-15 (lanes 2), were boiled at 100°C for 10 min and run on an SDS-PAGE gel. The gel was stained with Coomassie brilliant blue. Proteins transferred from the gel to a nitrocellulose membrane were probed with HLf-HRPO as described in the text. A boiled OM preparation was digested with proteinase K (30 to 60 U/ml; Sigma) at 37°C for 2 h and run on an SDS-PAGE gel; the gel was silver stained to demonstrate LPS. The electrophoresis and blotting conditions were described in the text. Arrows on the left indicate molecular mass standards (kilodaltons), and arrows to the right of the blot indicate the molecular masses (kilodaltons) of the HLf-HRPO-reactive bands.

Lf is a basic protein (pI 8.5) and may demonstrate electrostatic interactions with acidic molecules (21). Such a charge interaction between Lf and bacteria or their OMPs is less likely since NaCl (5 M) failed to dissociate the complex. The binding seems to be a protein conformation-dependent one, since chaotropic agents caused an effective uncoupling of the complex. Nonspecific hydrophobic interactions were reduced by incorporating Tween 20 in the test system.

The ability of bacteria to compete for iron within the host may influence pathogenesis (56). Aerobactin-producing *S. flexneri* strains may utilize host iron sources such as hemin or hematin (29), and a 101-kDa heme-binding protein has also been identified (53). Furthermore, the aerobactin-producing wild-type *S. flexneri* strains could remove iron from transferrin or Lf (29). Whether the Lf-binding proteins contribute to the bacterial iron acquisition mechanism in *S. flexneri* is under investigation.

It has been suggested that a two-component OmpR-EnvZ regulatory system is involved in the virulence of *S. flexneri* (5). Furthermore, a fucose-sensitive, carbohydrate-binding adhesin has been reported to be involved in the adherence of *S. flexneri* to colonic epithelia of guinea pigs (24). In the HLf molecule, fucose at α1-3 and at α1-6 linkages are important eucaryotic binding determinants (23, 38). Interestingly, fucose-containing HLf peptides are strong inhibitors of *S. flexneri* adherence to colonic epithelia (24).

In conclusion, we have demonstrated specific binding of Lf to *S. flexneri*. The Lf-binding OMPs in the bacterium are heat-modifiable and LPS-associated proteins which seem to be porins. The role of specific Lf interaction with *S. flexneri* in the pathogenesis of bacillary dysentery, however, remains to be elucidated.

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