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A Large Toxin from Pathogenic *Escherichia coli* Strains That Inhibits Lymphocyte Activation

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The mechanisms by which bacteria resist cell-mediated immune responses to cause chronic infections are largely unknown. We report the identification of a large gene present in enteropathogenic strains of *Escherichia coli* (EPEC) that encodes a toxin that specifically inhibits lymphocyte proliferation and interleukin-2 (IL-2), IL-4, and gamma interferon production in response to a variety of stimuli. Lymphostatin, the product of this gene, is predicted to be 366 kDa and shares significant homology with the catalytic domains of the large clostridial cytotoxins. A mutant EPEC strain that has a disruption in this gene lacks the ability to inhibit lymphokine production and lymphocyte proliferation. Enterohemorrhagic *E. coli* strains of serotype O157:H7 possess a similar gene located on a large plasmid. Loss of the plasmid is associated with loss of the ability to inhibit IL-2 expression while transfer of the plasmid to a nonpathogenic strain of *E. coli* is associated with gain of this activity. Among 89 strains of *E. coli* and related bacteria tested, *lifA* sequences were detected exclusively in strains capable of attaching and effacing activity. Lymphostatin represents a new class of large bacterial toxins that blocks lymphocyte activation.

Bacteria have evolved a number of mechanisms, including antiphagocytic factors, leukotoxins, and systems for iron chelation, to resist the nonspecific (innate) immune response of vertebrate hosts (25). In addition, several mechanisms to circumvent humoral immunity have been described, such as immunoglobulin A proteases and immunoglobulin-binding proteins. However, few bacterial factors that specifically interfere with cellular immune responses have been described (69). In addition, the mechanisms that allow certain bacterial pathogens to colonize hosts for prolonged periods remain obscure.

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of diarrhea among infants in developing countries. EPEC is one of the few known bacterial causes of chronic diarrhea (24, 34, 55). EPEC strains are characterized by their ability to induce profound cytoskeletal rearrangements in host cells that result in the formation of adhesion pedestals upon which the bacteria rest (47). This phenomenon is known as the attaching and effacing effect. Enterohemorrhagic *E. coli* (EHEC) strains, a subgroup of Shiga-toxin-producing *E. coli*, also have attaching and effacing activity (26, 64). We previously reported that EPEC produce and secrete a high-molecular-weight, protease-sensitive factor that selectively inhibits production of interleukin-2 (IL-2), IL-4, IL-5, and gamma interferon by human peripheral and lamina propria mononuclear cells and inhibits proliferation of these cells (35, 36, 41). This inhibitory effect was observed regardless of whether the cells were stimulated by phorbol esters, mitogens, CD3 cross-linking, or antigen. The effect was also seen in macrophage-depleted T-cell populations and in Jurkat cells, indicating that this activity did not require participation of cells other than lymphocytes. Despite the inhibition of lymphocyte function, these cells remain viable, and there is no evidence that they undergo apoptosis (35). We also reported that a cosmid clone isolated from an EPEC genomic library conferred upon a laboratory strain of *E. coli* the ability to produce a similar effect (36). The purpose of this study was to identify the genes responsible for this lymphocyte inhibitory factor (LIF) activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* E2348/69 (serotype O127:H6) is a classical EPEC strain isolated during an outbreak of infantile diarrhea and capable of causing diarrhea in adult volunteers (38). *E. coli* EFC1 was isolated from the feces of a healthy volunteer (46). *E. coli* EDL933 (serotype O157:H7) is an EHEC strain isolated from an outbreak of hemorrhagic colitis, while EDL933cu is a plasmid-cured derivative of that strain (63). *E. coli* C600-LK3 is a K-12 strain transformed with the large plasmid of strain EDL933, which had been marked with a Tn*801* transposon insertion (32). *E. coli* DH5α (Gibco BRL, Gaithersburg, Md.) was used for cloning standard plasmids, strain MC1061 (68) was used as a recipient for infection with lambda phage derivatives, strain DH5αλpir (43) was used to propagate suicide vectors, and strain HB101 (pRK2073) was used for triparental mating (14). A collection of enteric bacteria including various pathotypes of *E. coli* and related species is described in Table 1. All strains were stored at −80°C in 50% Luria broth (LB)–50% glycerol (vol/vol) and were grown in LB or on Luria plates. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; tetracycline, 15 μg/ml; chloramphenicol, 25 μg/ml; and nalidixic acid, 100 μg/ml.

Assays for localized adherence and attaching and effacing activities of EPEC were performed with HEp-2 cells as previously described (18).

Bacterial genetic techniques. *E. coli* MC1061 containing cosmid IV-8-A (36) was grown in LB medium supplemented with 0.2% maltose to a concentration of approximately 3×10^8 CFU/ml (optical density at 600 nm = 0.3) and infected with λ1105 at a multiplicity of infection of 0.3 as described (68). Transfectants were selected on medium containing kanamycin and tetracycline. Plasmids were recovered from pooled transfectants and were used to transform *E. coli* DH5α to kanamycin and tetracycline resistance.

DNA sequencing of plasmid templates subcloned from cosmids was performed at the University of Maryland Biopolymer Laboratory by using a model 373A sequencer (Applied Biosystems, Foster City, Calif.). Both nested deletions and primer-walking techniques were applied. A 1,791-bp *Eco*RI fragment was used as a gene probe to identify an additional cosmid from an independently constructed cosmid library of the same EPEC strain. Subclones from this cosmid (10-E-5) were used to complete the DNA sequence described herein.

To construct a *lifA* mutant, a 4,258-bp *Hin*dIII fragment internal to the *lifA* gene was cloned into pACYC184 (12). Digestion with *Xma*III and religation resulted in the excision of a 1,980-bp fragment. The *aphA-3* gene, together with upstream translational terminators and a downstream ribosome binding site and

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TABLE 1. Prevalence of *lifA* sequences in strains of *E. coli* and related organisms

Species	Strain	Pathotype[a]	Serotype or serogroup	Attaching and effacing activity	*lifA* probe results	Reference or source[b]
E. coli	13	EPEC	O111	+	+	30, 31, 39
E. coli	41	EPEC	O111	+	+	30, 31, 39
E. coli	45	EPEC	O111	+	+	30, 31, 39
E. coli	68	EPEC	O111	+	+	30, 31, 39
E. coli	71	EPEC	O111	+	+	30, 31, 39
E. coli	009-271082	EPEC	O111	+	+	30, 31, 49
E. coli	031-051082	EPEC	O111	+	+	30, 31, 49
E. coli	031-161182	EPEC	O111	+	+	30, 31, 49
E. coli	045-291182	EPEC	O111	+	+	30, 31, 49
E. coli	081-080883	EPEC	O111	+	+	30, 31, 49
E. coli	2309-77	EPEC	O111ab:H2	+	+	30, 31
E. coli	2430-78	EPEC	O111ab:NM	+	+	5, 16, 30, 31
E. coli	2198-77	EPEC	O111ab:NM	+	+	15, 16
E. coli	10	EPEC	O119	+	−	30, 31, 39
E. coli	22	EPEC	O119	+	−	30, 31, 39
E. coli	27	EPEC	O119	+	−	30, 31, 39
E. coli	51	EPEC	O119	+	−	30, 31, 39
E. coli	61	EPEC	O119	+	−	30, 31, 39
E. coli	77	EPEC	O119	+	−	30, 31, 39
E. coli	070-210183	EPEC	O119	+	−	30, 31, 49
E. coli	105-140583	EPEC	O119	+	−	30, 31, 49
E. coli	34	EPEC	O119	+	−	30, 31, 39
E. coli	0659-79	EPEC	O119:H6	+	−	5, 16, 30
E. coli	2450-80	EPEC	O119:H6	+	−	5, 30, 31
E. coli	2395-80	EPEC	O119:H6	+	−	5, 30, 31
E. coli	023-220982	EPEC	O126	+	+	30, 31, 49
E. coli	029-261082	EPEC	O126	+	+	30, 31, 49
E. coli	086-240583	EPEC	O127	+	−	30, 31, 49
E. coli	E2348/69	EPEC	O127:H6	+	+	17, 19, 37, 38
E. coli	050-250783	EPEC	O142	+	+	30, 31, 49
E. coli	012-050982	EPEC	O142	+	−	30, 31, 49
E. coli	042-060983	EPEC	O142	+	+	30, 31, 49
E. coli	851/71	EPEC	O142:H6	+	+	37, 62
E. coli	6	EPEC	O55	+	+	30, 31, 39
E. coli	9	EPEC	O55	+	+	30, 31, 39
E. coli	11	EPEC	O55	+	−	30, 31, 39
E. coli	30	EPEC	O55	+	+	30, 31, 39
E. coli	37	EPEC	O55	+	+	30, 31, 39
E. coli	55	EPEC	O55	+	+	30, 31, 39
E. coli	59	EPEC	O55	+	+	30, 31, 39
E. coli	73	EPEC	O55	+	+	30, 31, 39
E. coli	2087-77	EPEC	O55:H6	+	+	5, 16, 30, 31
E. coli	0660-79	EPEC	O55:H7	+	+	5, 16, 30
E. coli	0036-78	EPEC	O55:H7	+	+	5
E. coli	2362-75	EPEC	O55:NM	+	+	5, 30, 31
E. coli	38	EPEC	O86	+	−	30, 31, 39
E. coli	43	EPEC		+	+	30, 31, 39
E. coli	EDL931	EHEC	O157:H7	+	−	16, 64
E. coli	86-81	EHEC	O157:H7	+	−	16
E. coli	86-24	EHEC	O157:H7	+	−	27
E. coli	85-989	EHEC	O157:H7	+	−	CVD
E. coli	85-855	EHEC	O157:H7	+	−	CVD
E. coli	E3787	EHEC	O26:H11	+	+	B. Rowe
E. coli	85-707	EHEC	O26:H11	+	+	CVD
E. coli	85-0839	EHEC	O111:NM	+	+	CVD
E. coli	RDEC-1	REPEC	O15:NM	+	+	10, 11, 30, 70
E. coli	JM221	EAEC	O78:H33/35	−	−	42, 65, 66
E. coli	17-2	EAEC	O?:H2	−	−	50, 57, 66
E. coli	042	EAEC	O44:H18	−	−	66
E. coli	73-1	EAEC		−	−	CVD
E. coli	E12860/0	EIEC	O124:H−	−	−	59
E. coli	E20850/0	EIEC	O143:H−	−	−	59
E. coli	E50851/1	EIEC	O164:H−	−	−	59
E. coli	E5273/0	EIEC	O28ac:H−	−	−	16, 59
E. coli	E1181A	ETEC	O25:H42	−	−	CVD
E. coli	CFID499-2	ETEC	O6	−	−	CVD
E. coli	M408C1	ETEC	O6:H16	−	−	CVD

Continued on following page

TABLE 1—*Continued*

Species	Strain	Pathotype[a]	Serotype or serogroup	Attaching and effacing activity	*lifA* probe results	Reference or source[b]
E. coli	H10407	ETEC	O78:H11	−	−	16, 61
E. coli	E44324	UPEC	O15:H1	−	−	53
E. coli	CFT073	UPEC		−	−	46
E. coli	CFT325	UPEC		−	−	46
E. coli	CFT132	UPEC		−	−	46
E. coli	HS	NF		−	−	21, 37
E. coli	FN414	NF		−	−	46
E. coli	EFC4	NF		−	−	46
E. coli	EFC1	NF		−	−	46
E. coli	EFC2	NF		−	−	46
Citrobacter freundii	29219			−	−	ATCC
C. rodentium	4280			+	+	58
Hafnia alvei	13337			−	−	ATCC
H. alvei	10457			+	−	3
H. alvei	10790			+	−	3
H. alvei	19982			+	−	2
Salmonella enterica serovar *enteritidis*	10697			−	−	CVD
Salmonella serovar *enteritidis*	3319			−	−	CVD
Salmonella enterica serovar *typhi*	Ty2			−	−	13, 23
Salmonella enterica serovar *typhimurium*	14028			−	−	44, 45, 54
Yersinia enterocolitica	937			−	−	48
Yersinia pseudotuberculosis	527-2			−	−	J. Glenn Morris

[a] Pathotype refers to the following categories of pathogenic *E. coli*: REPEC, rabbit enteropathogenic *E. coli*; EAEC, enteroaggregative *E. coli*; EIEC, enteroinvasive *E. coli*; ETEC, enterotoxigenic *E. coli*; UPEC, uropathogenic *E. coli*; and NF, normal flora *E. coli* from the feces of healthy volunteers.
[b] CVD, Center for Vaccine Development; ATCC, American Type Culture Collection.

start codon, was cloned into this *Xma*III site from pUC18K (43), such that the downstream start codon was in frame with the 3′ end of the *lifA* gene. A *Sph*I-*Xba*I fragment containing this construction was then cloned into positive-selection suicide vector pCVD442 (17) and introduced into EPEC strain E2348/69 by triparental conjugation (14). Allelic exchange, with selection for loss of the suicide vector and wild-type allele on plates containing kanamycin and sucrose, was performed as previously described (20). The resulting mutant, UMD704, was verified by PCR with *Taq* DNA polymerase using 30 cycles (annealing at 50°C for 30 s, extension at 72°C for 2 min, denaturation at 94°C for 30 s). The primers for this PCR were Donne-280 (CGG AAC AGT AGG TTC ACC TTC) and Donne-281 (AGT GCC CGT GTT CTT GAA CTG), representing nucleotides 8088 to 8068 and 5863 to 5883 of the *lifA* sequence, respectively.

Colony hybridization was performed as previously described by using an internal *Eco*RI fragment representing nucleotides 3201 to 4991 of *lifA*, which had been labeled with [α-^{32}P]dATP by the random priming method (30).

PBMC stimulation and assay for cytokines. Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers. Whole blood was diluted 1:1 with phosphate-buffered saline (PBS) (pH 7.4) and was centrifuged (400 × *g*, 25 min, 21°C) over Histopaque 1077 (Sigma Chemicals Co., St. Louis, Mo.). PBMC were aspirated, washed in PBS, and centrifuged (200 × *g*, 10 min, 21°C). Cells were resuspended in RPMI 1640 medium (Gibco BRL) with 20 mM HEPES, 50 μg of gentamicin per ml, and 10% heat-inactivated fetal calf serum (Gemini Bioproducts Inc., Calabasas, Calif.) (pH 7.4) at a concentration of 10^6 cells per ml for all experiments. Cultures were maintained in a 5% CO_2 atmosphere for up to 2 days. Bacteria were grown overnight in LB medium, were centrifuged (4,000 × *g*, 10 min, 4°C), were resuspended in PBS, and were lysed in a French press at 20,000 lb/in^2. Unbroken cells were removed by centrifugation (1,000 × *g*, 10 min, 4°C). The protein concentration of the lysates was determined by the bicinchoninic acid method (Pierce, Rockford, Ill.).

After 2 hours of preincubation with bacterial lysates, PBMC were stimulated with the combination of 10 ng of phorbol myristate acetate (PMA) per ml and 5 μg of pokeweed mitogen (PWM) per ml. After further incubation for 6 h, PBMC were lysed in TRIzol reagent (Gibco BRL). Yeast tRNA (10 μg/sample) (Gibco BRL) was added, and mRNA was transcribed in 6.6 μl of reverse transcription buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$) (Promega, Madison, Wis.); 3.3 μl of dithiothreitol (50 mM); 1.5 μl of murine mammary lymphoma virus reverse transcriptase (200 U/ml) (Gibco BRL); 3.0 μl of oligo(dT)$_{16}$ (0.5 mg/ml) (Sigma); 1.0 μl of RNase inhibitor (40 U/μl) (Promega); 3.0 μl of acetyl-bovine serum albumin (1 mg/ml) (Gibco BRL); 1.5 μl of a mixture of dATP, dCTP, dGTP, and dTTP (1 mM each); and 1.5 μl of diethylpyrocarbonate-treated water for 1 h at 39°C. Five microliters of cDNA were amplified in a 45.0-μl PCR mixture consisting of 33.75 μl of H_2O, 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$); 4 μl of a mixture of dATP, dCTP, dTTP, and dGTP (1 mM each); 0.25 μl of Ampli*Taq* DNA polymerase (Gibco); and 1.0 μl of each primer (20 μM). The sequences of the primers were as follows: IL-2, 5′(ATG TAC AGG ATG CAA CTC CTG TCT T); IL-2, 3′(GTC AGT GTT GAG ATG ATG CTT TGA C); IL-4, 5′(AAC ACA ACT GAG AAG GAA ACC TTC); IL-4, 3′(GCT CGA ACA CTT TGA ATA TTT CTC); IL-5, 5′(GCT TCT GCA TTT GAG TTT GCT AGC T); IL-5, 3′(TGG CCG TCA ATG TAT TTC TTT ATT AAG); IL-8, 5′(ATG ACT TCC AAG CTG GCC GTG GCT); IL-8, 3′(TGA ATT CTC AGC CCT CTT CAA AAA); gamma interferon, 5′(CAG CTC TGC ATC GTT TTG GGT TCT); gamma interferon, 3′(TGC TCT TCG ACC TTG AAA CAG CAT); hypoxanthine phosphoribosyl transferase, 5′(GGA TTA TAC TGC CTG ACC AAG G); and hypoxanthine phosphoribosyl transferase, 3′(CGA GAT GTG ATG AAG GAG ATG G). Reactions were carried out in a Perkin-Elmer PCR cycler with 30 cycles consisting of a denaturing step at 94°C for 30 s, an annealing step at 60°C for 2 min, and an extension step at 72°C for 3 min. PCR products (15 μl/sample) were mixed with 2.0 μl of gel loading buffer per sample and were electrophoresed in a 3% agarose gel. Gels were stained in a 1% ethidium bromide solution and were examined under UV light.

To assay for cytokine secretion, PBMC were exposed to lysates from *E. coli* strains, stimulated 2 h later with PMA-PWM, and supernatants were harvested 24 h after stimulation. Cytokine concentrations in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA) according the manufacturer's specifications (Biosource International, Camarillo, Calif.) after a total of 24 h of incubation. Microtiter plates were read at 450 and 680 nm, and cytokine concentrations were determined with a linear-linear standard curve. Raw data were converted to mean values with standard errors, were normalized, and were expressed as percentages of the positive control by using the following formula: $(S-N)/(P-N) \times 100\%$, where S equals the cytokine concentration of the sample, P (positive control) equals the mean concentration of duplicate samples from stimulated cells, and N (negative control) equals the mean concentration of duplicate samples from unstimulated cells. Mean values pooled from duplicate samples of three independent experiments were compared using Student's *t* test. Two-tailed *P* values less than or equal to 0.05 were considered significant.

Cell proliferation assays. Caco-2 human colon carcinoma cells (56) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and gentamicin (50 μg/ml). PBMC and Caco-2 cells (2×10^5 per microtiter well) were incubated with bacterial lysates for 2 h and were stimulated with PMA (10 ng/ml) and PWM (5 μg/ml). Two days later, 1 μCi of [^{3}H]thymidine was added to each well, and the incubation was continued for an additional 4 h. PBMC and trypsin-treated Caco-2 cells were aspirated on fiberglass filter paper and were washed, and incorporated radioactivity was measured in a beta scintillation counter. Data were normalized and analyzed as described for cytokine assays.

Nucleotide sequence accession number. The nucleotide sequence reported herein was deposited in the EMBL database under accession no. AJ133705.

RESULTS

Identification and characterization of *lifA*. To localize the gene(s) encoding the LIF, we subjected a cosmid clone encoding LIF activity to mutagenesis by using a minitransposon.

Recombinant *E. coli* strains containing cosmids with transposon inserts were tested for the ability to inhibit expression of IL-2 mRNA by stimulated PBMC and the locations of the transposon inserts were mapped by restriction endonuclease digestion and PCR. All 15 transposon insertions that disrupted LIF activity mapped to a 9-kb region of the cosmid. In contrast, all 27 transposon insertions that retained LIF activity mapped outside of this region. DNA sequencing of the region revealed an open reading frame (ORF) spanning 9,669 bp, which we designate *lifA*. A putative ribosome-binding site was identified 8 bp upstream of the start codon. The *lifA* ORF is predicted to encode a protein of 365,950 Da. The size of the predicted protein is compatible with our previous finding that maximum LIF activity is associated with a fraction containing proteins greater than 100 kDa. A search of the available databases with Gapped BLAST (4) revealed significant similarity (28% identical, 47% similar residues) over its entire length with a hypothetical protein of unknown function predicted to be encoded by ORF L7095 on the large plasmid of EHEC O157:H7 strains EDL933 and RIMD 0509952 (8, 40). This finding is compatible with our earlier observation that EDL933 has LIF activity (36). In addition, a stretch of amino acids near the amino terminus bears significant similarity to a region of approximately 500 amino acids at the amino terminus of the large clostridial cytotoxins. These cytotoxins include toxin A and toxin B of *Clostridium difficile*, lethal toxin of *Clostridium sordellii*, and alpha toxin of *Clostridium novyi*. The large clostridial cytotoxins catalyze the glycosidation of critical threonine residues in members of the Ras family of small GTPases, thereby inactivating them (1, 67). It has previously been shown that a recombinant protein consisting of the amino-terminal 546 amino acids of *C. sordellii* lethal toxin, the domain with which *lifA* has homology, possesses full enzymatic activity and is capable of glucosidating Ras and Rho in vitro (28). Finally, the protein predicted by *lifA* bears significant homology to two hypothetical proteins encoded by adjacent ORFs from the *Chlamydia trachomatis* genome (60).

Construction and characterization of a *lifA* mutant of EPEC. To determine whether the product of *lifA* is required for the LIF activity of wild-type EPEC, we constructed a mutant of EPEC strain E2348/69 that has a 1,980-bp internal deletion of the *lifA* sequence. The mutation was confirmed by PCR (Fig. 1A). We analyzed the mutant, designated UMD704, for phenotypes characteristic of EPEC. Like the wild-type EPEC strain from which it was derived, the *lifA* mutant exhibited autoaggregation when grown in tissue culture medium (data not shown), was capable of localized adherence to epithelial cells, and was able to induce the attaching and effacing effect in infected cells (Fig. 1B). Thus, the disruption of *lifA* did not affect known EPEC virulence attributes. To assess LIF activity, the levels of IL-2, IL-4, IL-5, and gamma interferon messenger RNA in stimulated PBMC were assayed by reverse transcription-PCR. The levels of mRNA for IL-2, IL-4, and gamma interferon were markedly reduced in a dose-dependent fashion when the cells were incubated in the presence of lysates from the wild-type EPEC strain. The reduction for IL-5 mRNA was less pronounced and was only observed in high concentrations of wild-type EPEC lysate. In the presence of lysates from the *lifA* mutant or with lysates from a commensal *E. coli* strain, no lymphokine inhibition was observed. The decrease in mRNA concentrations of IL-2, IL-4, and gamma interferon suggests a transcriptional regulation of lymphokine expression. In contrast, there was no detectable effect on IL-8 mRNA levels (Fig. 2A) even in the highest concentrations of bacterial lysates tested. To quantitate the decrease in lymphokine expression, cell supernatants were assayed after 24 h of incubation by using ELISA. IL-2, IL-4, and gamma interferon protein concentrations were reduced in a dose-dependent fashion in cells treated with lysates from wild-type EPEC in comparison to cells treated with lysates from the *lifA* mutant and from the commensal *E. coli* strain (Fig. 2B). The differences between the wild type and *lifA* mutant in ability to suppress IL-2, IL-4, and gamma interferon protein expression were statistically significant at concentrations greater than or equal to 2.5, 25, and 5 μg/ml, respectively. There was no significant difference between the wild-type strain and the *lifA* mutant in ability to suppress IL-5 protein expression, although there appeared to be some suppression at higher doses. There was no difference between the *lifA* mutant strain and the commensal strain in ability to suppress expression of IL-2, IL-4, IL-5, or gamma interferon at any dose, with one exception. At doses less than or equal to 5 μg/ml, there was significantly more IL-2 produced by cells exposed to lysates from the *lifA* mutant than by cells exposed to lysates from the commensal strain.

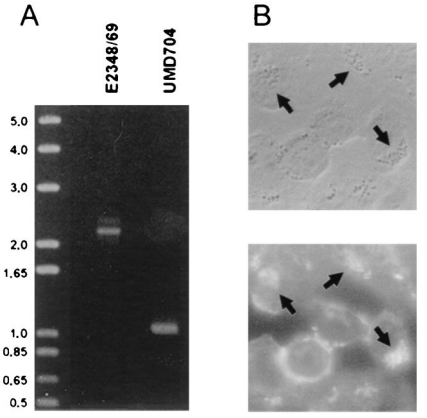
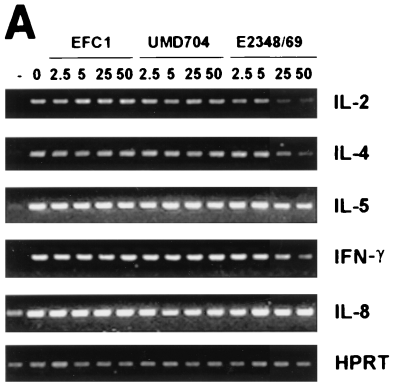


FIG. 1. A *lifA* mutant of EPEC retains the ability to perform localized adherence and attaching and effacing activities. By allelic exchange, the wild-type *lifA* locus of EPEC strain E2348/69 was replaced with an allele that contains an 1,890-bp deletion, at which site a nonpolar kanamycin resistance cassette was inserted, to create the *lifA* mutant strain UMD704. (A) Agarose gel electrophoresis of the products of a PCR targeting a portion of the *lifA* gene. The product from mutant strain UMD704 is approximately 1,130-bp smaller than that from the wild-type strain E2348/69, confirming the replacement of the 1,980-bp fragment with the 850-bp *aphA-3* gene cassette. The sizes (in kilobase pairs) of molecular standards are indicated on the left. (B) Phase-contrast micrograph (top) and corresponding fluorescein isothiocyanate-phalloidin fluorescence micrograph (bottom) of HEp-2 cells infected the *lifA* mutant UMD704. Arrows show microcolonies of bacteria associated with accumulations of filamentous actin that are characteristic of the localized adherence and attaching and effacing phenotypes.

To determine whether the product of *lifA* is required for inhibition of lymphocyte proliferation, we measured the effect of *E. coli* lysates on incorporation of [^{3}H]thymidine into stimulated PBMC. PBMC proliferation was markedly inhibited in a dose-dependent manner by the cell lysate of wild-type EPEC, but not by the lysates of the commensal *E. coli* strain or the *lifA* mutant (Fig. 3A). The difference between the wild-type strain and the *lifA* mutant was statistically significant at all concentrations of bacterial lysate tested. In contrast, none of the lysates from the three bacterial strains tested inhibited proliferation of human intestinal Caco-2 cells (Fig. 3B). In these experiments, there was a large variation in the effect of lysates from the commensal *E. coli* strain on Caco-2 proliferation, due in part to the relatively small difference in proliferation between unstimulated Caco-2 cells and those stimulated with PWM-PMA. However, the results with the wild-type strain and



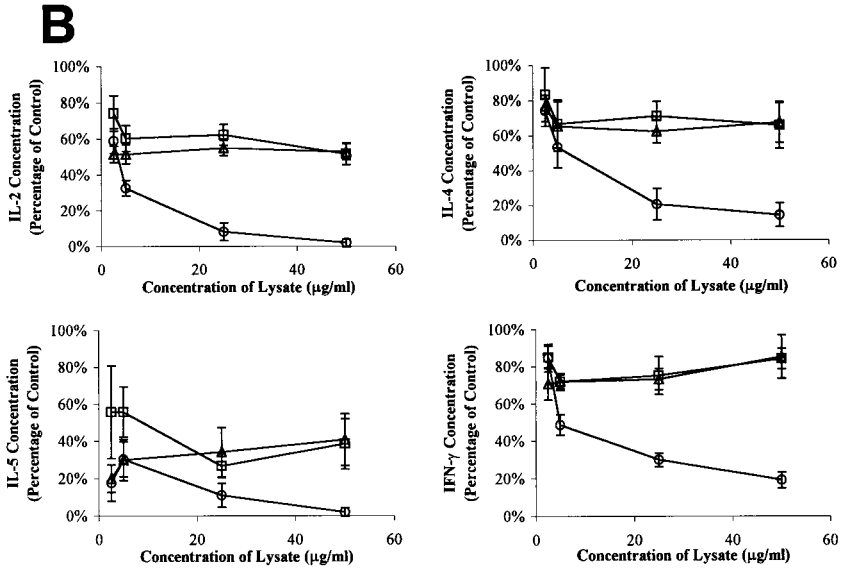


FIG. 2. Lymphostatin is required for EPEC-mediated inhibition of lymphokine expression by PBMC. (A) Effect of bacterial lysates on lymphokine mRNA expression. PBMC were incubated in the absence of bacterial lysates and were left unstimulated (−) or exposed to 0, 2.5, 5, 25, or 50 μg/ml of bacterial lysates from commensal *E. coli* EFC1, *lifA* mutant EPEC strain UMD704, or wild-type EPEC stain E2348/69 as indicated and were stimulated with PMA-PWM. Expression of IL-2, IL-4, IL-5, gamma interferon (IFN-γ), IL-8, and hypoxanthine phosphoribosyltransferase mRNA were assessed by reverse transcription-PCR. (B) Effect of bacterial lysates on lymphokine protein expression. PBMC were incubated with lysates from wild-type EPEC strain E2348/69 (circles), EPEC *lifA* mutant UMD704 (squares), or commensal *E. coli* EFC1 (triangles) and were stimulated as indicated in the legend to panel A. Values were calculated as described in Materials and Methods as percentages of concentrations measured in cells stimulated in the absence of lysates and are pooled from duplicate values of three independent experiments. Error bars represent standard errors of the means (SEMs). The absolute mean values (± SEMs) for cells stimulated in the absence of lysates were 4.38 ± 1.18 ng/ml for IL-2, 37.2 ± 4.8 pg/ml for IL-4, 99.7 ± 34.6 pg/ml for IL-5, and 1.84 ± 0.25 ng/ml for gamma interferon.

the *lifA* mutant were very similar and reproducible, indicating that the product of this gene has no effect on Caco-2 cell proliferation. Given the sequence homology between the product of *lifA* and the large clostridial cytotoxins, which disrupt the actin cytoskeleton and cause cell rounding by inactivating Rho family GTPases (67), we investigated whether EPEC lysates had any effect on actin distribution in tissue culture cells. We observed no effect of lysates of either the wild-type EPEC strain or the *lifA* mutant on the distribution of actin in HEp-2 cells or on cell morphology as assessed by fluorescence microscopy using fluorescein isothiocyanate-phalloidin (data not shown). Since the *lifA* gene product specifically inhibits lymphokine production and lymphocyte proliferation, we have named this protein lymphostatin.

Prevalence of *lifA* sequences among enteric bacteria and correlation with LIF activity. To determine the distribution of the *lifA* gene among enteric bacteria, we used a 1,791-bp internal *Eco*RI fragment as a DNA probe to detect *lifA* sequences in a panel of *E. coli* strains and related species by colony hybridization. A strong positive signal was detected in 35 of 60 strains of *E. coli* and related species capable of attaching and effacing activity, but was detected in 0 of 29 strains incapable of attaching and effacing ($P < 0.001$, χ^{21} test) (Table 1). Among EPEC strains, more O111 strains (13 of 13) and O55 strains (11 of 12) than O119 strains (0 of 12) were probe positive. All five strains tested and found to have LIF activity yielded a strong hybridization signal with the *lifA* probe. In contrast, all five strains tested that lacked LIF activity, including O119:H6 EPEC strain 0659-79, were probe negative ($P = 0.008$, Fisher's exact test). As expected, the EHEC O157:H7 strains tested did not hybridize with the probe, since there is insufficient DNA similarity between *lifA* and the ORF L7095 on the large plasmid of EHEC to yield a positive probe signal under stringent conditions. To determine whether ORF L7095 of EHEC could potentially encode the LIF activity found in O157:H7 strains, we compared the ability of EHEC strain EDL933 to inhibit IL-2 expression with that of a plasmid-cured derivative of that strain (EDL933cu). Whereas lysates of strain EDL933 inhibited IL-2 production by PBMC in a dose-dependent fashion, there was no effect of lysates of strain EDL933cu on IL-2 expression (Fig. 4). The difference in ability to suppress IL-2 expression between strains EDL933 and EDL933cu were statistically significant at lysate protein concentrations greater than or equal to 5 μg/ml. Moreover, lysates of a recombinant *E. coli* K-12 strain into which the large plasmid of EHEC had been introduced inhibited IL-2 production while lysates of the same strain without the plasmid did not. This difference was significant at concentrations of 25 μg/ml and higher. This result indicates that a factor encoded on the

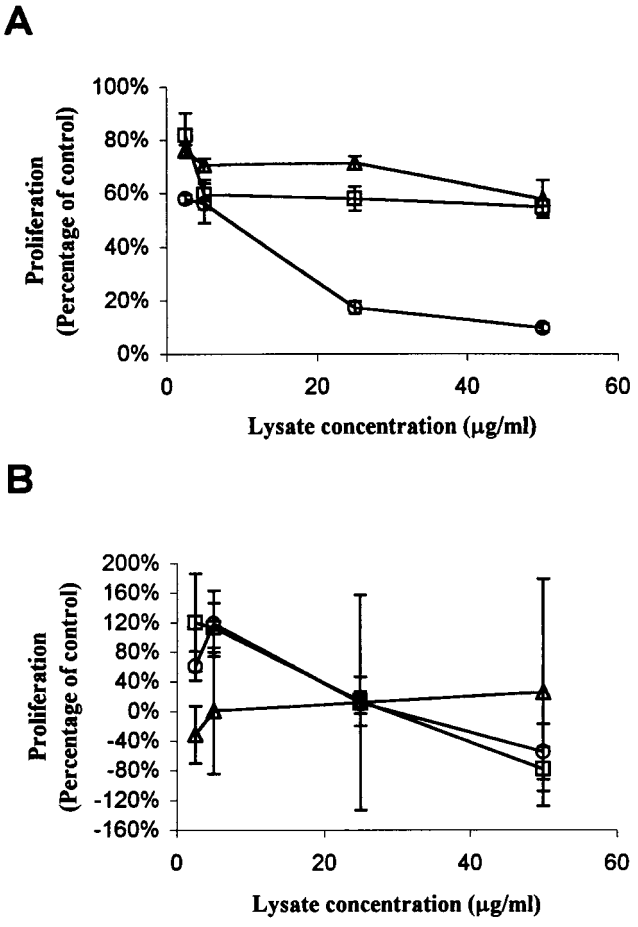


FIG. 3. Lymphostatin is required for inhibition of lymphocyte but not epithelial cell proliferation by EPEC. PBMC (A) or Caco-2 cells (B) were incubated with lysates from wild-type EPEC strain E2348/69 (circles), EPEC *lifA* mutant UMD704 (squares), or commensal *E. coli* strain EFC1 (triangles), stimulated with PMA-PWM, and incorporation of thymidine was measured by scintillation counting after 2 days. Values were calculated as percentages of controls as described in Materials and Methods. Data shown represent the mean percentages ± SEMs from quadruple determinations of two independent experiments.

EHEC plasmid is required for LIF activity in *E. coli* O157:H7. The best candidate for this factor is the product of ORF L7095.

DISCUSSION

In earlier studies, we described a LIF produced by EPEC and related pathogens that blocks cytokine expression and lymphocyte proliferation without inducing apoptosis or otherwise killing cells (35, 36, 41). In the present study, we report the identification of the *lifA* gene of EPEC strain E2348/69 isolated from a cosmid clone that expresses inhibitory activity. To test the role of *lifA* in lymphocyte activation, we generated a nonpolar, in-frame mutation of the gene. Lysates of this mutant lacked the ability of wild-type EPEC lysates to inhibit expression of IL-2, IL-4, and gamma interferon mRNA and protein in mitogen-stimulated lymphocytes. The expression of IL-8 was unaffected. Furthermore, lysates from wild-type EPEC caused a dose-dependent decrease in lymphocyte proliferation; a normal proliferative response was observed in mitogen-stimulated lymphocytes exposed to the lysates from the *lifA* mutant. Epithelial cells were unaffected and did not show a decreased proliferative response or cytoskeletal disruption. We therefore conclude that *lifA* is required for the inhibitory activity observed in mitogen-stimulated lymphocytes exposed to lysates of wild-type EPEC.

The *lifA* gene spans 9,669 bp and is thus the largest reported gene in *E. coli*. Its putative product, lymphostatin, has a molecular mass of 366 kDa, making it one of the largest bacterial toxins known. Lymphostatin bears significant homology to the large clostridial cytotoxins in a stretch of approximately 500 amino acid residues located near the amino terminus. This region of the *C. difficile* toxin B possesses full enzymatic activity, but only when microinjected into cells, suggesting that the rest of the molecule, with which lymphostatin lacks homology, contains sequences for cell binding and translocation (29). Interestingly, in EPEC and *Clostridium*, this conserved stretch of DNA contains nucleotides encoding for a "D-X-D motif". This D-X-D motif has been implicated to be the enzymatically active site of a glucohydrolase-glycosyltransferase activity (9). Large clostridial cytotoxins have been shown to glycosylate small GTP-binding proteins, like Rho, Rac, or Cdc42, at a conserved threonine site, thereby inactivating them (67). Glycosylation of small GTP-binding proteins leads to profound changes in the actin cytoskeleton with characteristic rounding of epithelial cells. However, in our experiments, *lifA* was associated with a profound effect on lymphokine expression but did not cause changes in the epithelial cell cytoskeleton, suggesting a different mechanism of action. It is possible that lymphostatin glycosylates other small GTP-binding proteins, exhibits specificity for host cell receptors, or works through an unidentified mechanism altogether. Elucidation of the mechanism by which lymphostatin functions awaits further investigation. Although we have shown that the *lifA* gene is required for inhibitory activity, we have not proven that lymphostatin is sufficient for this activity. To do so will require purified protein, which has been difficult to obtain since it appears to be produced in small amounts and to be labile (data not shown).

Colony hybridization with an internal *lifA* probe identified the presence of a similar gene in other EPEC strains, in EHEC strains of serotypes other than O157:H7, and in *Citrobacter*

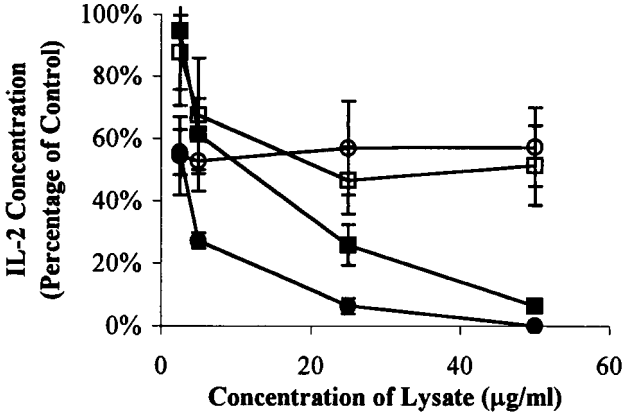


FIG. 4. The large plasmid of *E. coli* O157:H7 encodes a factor that is required for inhibition of IL-2 expression by PBMC. PBMC were exposed to various concentrations of bacterial lysates from EHEC wild-type strain EDL933 (closed circles), the plasmid-cured derivative EDL933cu (open circles), *E. coli* K-12 strain C600 (open squares), or strain C600-LK3 (transformed with the large EHEC plasmid, closed squares). Cells were stimulated 2 h later with the PMA-PWM combination, and supernatants were harvested after a total of 24 h. Concentrations of IL-2 in the supernatant were measured by ELISA. Values were calculated as described in Materials and Methods as percentages of concentrations measured in cells stimulated in the absence of lysates and are pooled from duplicate values of three independent experiments. Error bars represent SEMs. The absolute mean value (±SEM) for cells stimulated in the absence of lysates was 3.78 ± 1.48 ng/ml.

rodentium. Thus, DNA sequences similar to *lifA* are common in bacteria that perform the attaching and effacing lesions on host epithelial cells, but were not found in other *E. coli* and related organisms. The attaching and effacing effect is encoded by a large pathogenicity island known as the locus of enterocyte effacement. However, at least in EPEC strain E2348/69 and EHEC strain EDL933, *lifA* is not part of this pathogenicity island (22, 52).

In the present study, we found that EHEC O157:H7 strains do not yield a positive signal in hybridization experiments, yet we determined that the large plasmid of strain EDL933 is necessary and sufficient for lymphocyte inhibition. The most likely candidate for the gene encoding the inhibitory activity is ORF L7095, which is predicted to encode a protein that has similarity (28% identical, 47% similar residues) to lymphostatin. This low level of similarity possibly accounts for the lack of a positive hybridization signal. Studies are in progress to isolate and clone this gene to determine whether alone it can confer inhibitory activity to a recombinant *E. coli* strain.

Interestingly, a notable characteristic of attaching and effacing pathogens is their ability to colonize their hosts for prolonged periods. EPEC is a leading cause of both acute and chronic diarrhea in developing countries, while O157:H7 EHEC strains cause life-threatening hemorrhagic colitis and hemolytic-uremic syndrome and can colonize patients for prolonged periods of time (6, 7, 33, 51). One potential consequence of lymphostatin expression might be the suppression of an immune response to the bacteria, prolonging the infection and increasing the opportunity for transmission to new hosts. Enteric bacterial products similar to lymphostatin could also play a role in deregulating cytokine production in chronic inflammatory syndromes such as ulcerative colitis and Crohn's disease. The further finding of sequences related to lymphostatin in *Chlamydia* suggests that similar proteins may contribute to chronic infections that lead to blindness, infertility, and perhaps atherosclerosis.

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J.-M.A.K. and I.C.A.S. contributed equally to this work.

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