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Products of Enteropathogenic *Escherichia coli* Inhibit Lymphocyte Activation and Lymphokine Production

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The aim of this study was to determine whether products of enteric bacteria are able to regulate lymphocyte activation and cytokine production. Whole bacteria and bacterial lysates from different strains of *Escherichia coli* were tested for their ability to inhibit cytokine production by peripheral blood mononuclear cells as determined by reverse transcription-PCR, Northern (RNA) blotting of cellular RNA, or enzyme-linked immunosorbent assay for cytokine protein. Lysates from two pathogenic strains of *E. coli*, enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli*, inhibited mitogen-stimulated expression of interleukin-2 (IL-2), IL-4, IL-5, and gamma interferon. IL-1β, IL-6, IL-8, IL-10, IL-12, and Rantes mRNA expression was not affected. The inhibitory activity was dose dependent, protease and heat sensitive, nondialyzable, and not due to cellular toxicity. The inhibitory activity remained in EPEC strains having mutations in known virulence factors. Nonpathogenic *E. coli* HB101 transformed with a 22-kb cosmid clone derived from EPEC chromosomal DNA expressed the inhibitory activity. Thus, certain strains of pathogenic *E. coli* express a protein or proteins encoded by chromosomal genes that selectively inhibit lymphocyte activation and lymphokine production. Therefore, immunosuppressive factors produced by pathogenic bacteria could be important in modifying gastrointestinal immune responses in enteric bacterial infections or gastrointestinal autoimmune diseases.

The human gastrointestinal tract contains a large number of lymphoid and myeloid cells, many of which are activated and which play important host defense roles (20, 23, 35, 39). The continuous activation of these cells is thought to be due in part to the stimulatory effect of bacterial antigens and bacterial lipopolysaccharides normally present in the gastrointestinal lumen (1, 5, 22). In addition, numerous macromolecules derived from the diet can potentially stimulate the immune system, although the usual outcome of this stimulation is immune tolerance (3, 26). The mechanisms by which the continuous low-grade activation of the gastrointestinal immune system is regulated are not well understood. Enteric bacterial pathogens that evade normal host defense mechanisms by attachment to enterocytes, invasion of the mucosa (20), or production of toxins (31) normally elicit a prompt immune and inflammatory response by the local immune system (23). It is currently thought that the interaction of the normal enteric flora and enteric pathogens with the gastrointestinal immune system has for the most part a stimulatory effect. However, it is possible that individual components of the enteric flora might also play a more complex role in regulating the activity of the mucosal immune system. To address this possibility, the specific objective of this study was to determine whether components of enteric bacteria contain factors capable of inhibiting immune responses, as assessed by lymphocyte proliferation and cytokine expression. The results demonstrate the presence of a novel inhibitory activity present in certain strains of *Escherichia coli* that may potentially play a role in gastrointestinal infections and other gastrointestinal inflammatory diseases.

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MATERIALS AND METHODS

Table 1 lists bacterial strains used in this study. Prior to experiments, bacteria were centrifuged (400 × *g*, 10 min, 4°C) and washed in phosphate-buffered saline (PBS) buffer (0.01 M sodium phosphate, 0.15 M NaCl [pH 7.2]). The bacterial pellet was resuspended in PBS, and the concentration of bacteria was estimated by A_{600}.

Inactivation of bacteria. Inactivation of bacteria was carried out using a method described by Jansson and Kronvall (16). Pathogenic and nonpathogenic bacteria described in Table 1 were grown overnight, washed twice in PBS, resuspended in 1.5% (vol/vol) formalin in PBS, and incubated for 1.5 h at 23°C. After this incubation, bacteria were washed again in PBS and heat inactivated for 5 min at 80°C. Bacterial concentration was estimated at 600 nm, and the suspensions were stored at −70°C until further use.

Bacterial lysates. Bacteria were resuspended in approximately 3.5 ml of PBS and lysed in a French pressure cell (2×10^4 lb/in^2). The lysate was centrifuged (1,000 × *g*, 10 min, 4°C) to remove cell debris and whole bacteria. Protein concentration was determined by a colorometric assay (Bio-Rad Laboratories, Richmond, Calif.), using albumin as a standard. Samples were stored at −70°C until further use.

Cell culture and activation. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers. Whole blood was subjected to Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.) centrifugation (400 × *g*, 30 min, 23°C). Mononuclear cells were aspirated, washed in PBS, and centrifuged (200 × *g*, 10 min, 23°C). The cell preparation was resuspended in complete RPMI 1640 (GIBCO BRL Inc., Grand Island, N.Y.), containing 2 mM glutamine, 1 mM sodium pyruvate, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 7.4, and 10% fetal calf serum (Gemini Bioproducts, Inc., Calabasas, Calif.) in a 5% CO_2 atmosphere. Cells were counted in a hemacytometer. For reverse transcription-PCR (RT-PCR) experiments, a cell concentration of 1.5×10^6/ml was used; for Northern (RNA) blot experiments 2×10^7/ml was used. All experiments performed with French press lysates contained gentamicin (50 μg/ml) in the culture medium. For experiments with live bacteria, culture media containing gentamicin (50 μg/ml) were used after an initial incubation period of 2 h. PBMCs were stimulated with either phorbol myristate acetate (PMA) (Sigma; 20 ng/ml), pokeweed mitogen (PWM) (GIBCO; dilution, 1:100), phytohemagglutinin (PHA) (Sigma; 10 μg/ml), or anti-CD3 (OKT-3; 1:1,000 final concentration), which were added after 2 h of culture.

Lymphocyte proliferative responses were tested using PWM (1:100 dilution), which was added to 2×10^5 cells per microculture well. On day 3 cells were pulsed for 4 h with 1 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, Ill.). Cells were harvested on fiberglass filter paper. Radioactivity was measured in a beta scintillation counter. Determinations were made in triplicate. Data were

TABLE 1. *E. coli* strains used in this study

Strain	Characteristics	Source[a]	Reference
EFC1	Isolate from feces of healthy volunteer	UMAB	24
FN414	Isolate from feces of healthy volunteer	R. Hull	24
17-2	Serotype O3:H2 enteroaggregative strain	J. Nataro	27
H10407	Serotype O78:H1 enterotoxigenic strain	C. Denecke	7
CFT325	Isolate from blood of patient with acute pyelonephritis	UMAB	24
EDL933	Serotype O157:H7 EHEC strain isolate	A. O'Brien	37
HB101	*E. coli* K-12 × *E. coli* B hybrid, highly transformable	GIBCO	
EPEC			
0659-79	Serotype O119:H6	CDC	7
2087-77	Serotype O55:H6	CDC	7
2430-78	Serotype O111:NM	CDC	7
E2348/69	O127:H6 isolate from outbreak of infant diarrhea	B. Rowe	7
MAR 20	Derivate of E2348/69 cured of the EPEC adherence factor plasmid during passage in the laboratory	UMAB	2
JPN15	Derivate of E2348/69 cured of the EPEC adherence factor plasmid during passage in a volunteer	UMAB	17
31-6-1(1)	*bfpA*::Tn*phoA*; unable to produce bundle-forming pilus, deficient in localized adherence	UMAB	6, 8
CVD206	Δ*eaeA*; unable to produce intimin, deficient in intimate attachment	UMAB	10
UMD864	Δ*eaeB*; deficient in intimate attachment and signal transduction	UMAB	11
16-9-1(1)	*dsbA*::Tn*phoA*; unable to produce periplasmatic disulfide bond oxidoreductase, deficient in localized adherence	UMAB	6, 40
14-2-1(1)	*cfm*; deficient in signal transduction	UMAB	6, 30
27-3-2	*cfm*; deficient in signal transduction	UMAB	6, 30
30-5-1(3)	Tn*phoA* insertion in undefined locus; deficient in invasion	UMAB	6

[a] UMAB, University of Maryland at Baltimore; CDC, Centers for Disease Control and Prevention.

expressed as mean counts per minute. Viability of isolated PBMCs was determined by trypan blue exclusion assay.

RNA isolation. Total RNA was isolated from PBMCs using TRIzol Reagent (GIBCO BRL) according to the manufacturer's specifications. In order to quantitatively precipitate all RNA from small numbers of lymphocytes, 20 μg of yeast tRNA was added as a carrier to each sample prepared for RT-PCR (15). Since the amount of RNA obtained from lymphocytes was not quantitated, comparisons of lymphokine RNA were always made for samples containing identical numbers of lymphocytes (14). Precipitated nucleic acids were washed in 75% ethanol, dried, and resuspended in 12.5 μl of H_2O.

RT-PCR. RT-PCR for analysis of cytokines was performed as previously described (15). The reverse transcription buffer contained 1.5 μl of Moloney murine leukemia reverse transcriptase (M-MLV RT) (200 U/ml; GIBCO), 1 μl of RNasin (40 U/μl; GIBCO), 6.6 μl of 5× RT-buffer (GIBCO; 250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$), 3.3 μl of dithiothreitol (50 mM; GIBCO), 3 μl of acetyl-bovine serum albumin (1 mg/ml; GIBCO), 1.5 μl of a mix of dATP, dTTP, dCTP, and dGTP (1 mM each; Boehringer Mannheim, Indianapolis, Ind.), and 1.5 μl of diethylpyrocarbonate (DEPC)-treated H_2O. The reaction was carried out at 39°C for 1 h in a Perkin-Elmer 480 DNA Thermal Cycler (Perkin-Elmer Corp., Norwalk, Conn.).

Five microliters of reverse-transcribed RNA was added to 45.4 μl of PCR buffer consisting of 30.75 μl of DEPC-treated H_2O, 5.4 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 4 μl of a mix of dATP, dTTP, dCTP, dGTP (1 mM each), 0.25 μl of Ampli-Taq DNA polymerase (GIBCO), and 2.5 μl each of 20 μM 5′ and 20 μM 3′ primers. Each sample was overlaid with a drop of mineral oil (Fisher Scientific, Fair Lawn, N.J.).

PCR primers were chosen to span at least one intron. Table 2 lists the primers used in this study (sense and antisense).

Standard PCR consisted of 30 cycles and was carried out in a Perkin-Elmer 480 DNA Thermal Cycler. Each cycle consisted 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.

TABLE 2. Primers used in this study

Primer	Sequence	Reference
β-Actin	5′-GTGGGGCGCCCCAGGCACCA	15
	3′-CTCCTTAATGTCACGCACGATTTC	
IL-1β	5′-ATGGCAGAAGTACCTGAGCTCGCC	15
	3′-GCTTTTTTGCTGTGAGTCCCGGA	
IL-2	5′-ATGTACAGGATGCAACTCCTGTCTT	15
	3′-GTCAGTGTTGAGATGATGCTTTGAC	
IL-4	5′-ATGGGTCTCACCTCCCAACTGCT	15
	3′-GTTTTCCAACGTACTCTGGTTGGC	
IL-5	5′-GCTTCTGCATTTGAGTTTGCTAGCT	15
	3′-TGGCCGTCAATGTATTTCTTTATTAAG	15
IL-6	5′-AACTCCTTCTCCACAAGCG	15
	3′-TGGACTGCAGGAACTCCTT	15
IL-8	5′-ATGACTTCCAAGCTGGCCGTGGCT	15
	3′-TGAATTCTCAGCCCTCTTCAAAAA	
IL-10	5′-CTGAGAACCAAGACCCAGACATCAAG	38
	3′-CAATAAGGTTTCTCAAGGGGCTGGGTC	
IL-12	5′-ATGTGTCACCAGCAGTTGGTCATCTCT	13
	3′-ATAATTCTTGGCCTCGCATCTTAG	
IFN-γ	5′-CAGCTCTGCATCGTTTTGGGTTCT	15
	3′-TGCTCTTCGACCTTGAAACAGCAT	
Rantes	5′-ATGAAGGTCTCCGCGGCACGCCTCGCTGTC	34
	3′-CTAGCTCATCTCCAAAGAGTTGAT	

Interleukin-12 (IL-12) was amplified in 40 cycles, followed by an additional extension step at 72°C for 7 min for all investigated cytokines.

PCR products (18 μl) were mixed with 2 μl of sample loading buffer containing bromphenol blue and electrophoresed on a 3% agarose gel with 1 μg of ethidium bromide per ml. PCR products were evaluated as ethidium bromide-stained bands under UV light. The molecular weight DNA standard was a 1-kb DNA ladder (GIBCO).

Northern blot analysis. RNA samples (10 μg) from 2×10^7 PBMCs were dissolved in denaturing buffer (50% deionized formamide, 6% formaldehyde, 10 mM sodium phosphate buffer, 0.5 mM EDTA [pH 7.4]) and heated at 65°C for 15 min. The samples were mixed with 5 μl of loading buffer (50% glycerol, 1 mM EDTA [pH 8.0], 0.25% bromphenol blue, 0.25% xylene cyanol FF) and loaded on a 1% agarose gel containing 1.1 M formaldehyde. After completion of electrophoresis, total RNA was bound to nitrocellulose by capillary transfer and fixed by UV cross-linking (Stratagene, LaJolla, Calif.). The prehybridization and hybridization solution consisted of the following: 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), 100 μg of denatured, fragmented salmon sperm DNA per ml, and 50% deionized formamide. Prehybridization was carried out overnight at 42°C.

Hybridization was carried out with a PCR-generated probe specific for IL-2. One hundred nanograms of amplified DNA was labeled with [^{32}P]dCTP (Amersham Corp.) by a random primer method (Sigma). After hybridization at 42°C overnight, filters were washed in 1× SSC–1% SDS at room temperature once and three times in 0.1× SSC–0.2% SDS at 65°C. The filters were exposed to Kodak XOmat-AR film (Kodak, Rochester, N.Y.) at −70°C with an intensifier screen.

ELISA for IL-2. Culture supernatant IL-2 was measured by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Inkstar, Stillwater, Minn.). The minimum detectable concentration was 0.1 IU/ml. Microtiter plates were read at 450 nm, and the concentration of IL-2 in the supernatant was determined with a linear-linear standard curve. Determinations were carried out in duplicate.

Cosmid clones from EPEC. An enteropathogenic *E. coli* (EPEC) cosmid library was derived through a partial digestion of the bacterial chromosome of MAR20 (plasmid-cured derivative of EPEC E2348/69) with *Pst*I. This digest was ligated into the commercially available cosmid vector pHC79 (GIBCO), packaged into lambda particles, and used to transfect *E. coli* HB101. Single clones were isolated on Luria agar containing tetracycline (15 μg/ml) and stored in 50% Luria-Bertani broth–50% glycerol in microtiter wells at −70°C.

RESULTS

Stimulation of cytokine mRNA by whole bacteria and bacterial lysates. Initially, experiments were carried out to determine the effect of whole bacteria on cytokine mRNA expression in culture with PBMCs. Whole bacteria (5×10^5) were incubated together with 1.5×10^6 PBMCs for an initial 2 h of stimulation. To prevent bacterial overgrowth of the cultures, medium was then changed to RPMI 1640 with gentamicin (50 μg/ml) to kill extracellular bacteria. After 8 h of incubation, the expression of cytokines by PBMCs was determined by RT-PCR of RNA isolated from lymphocytes. In the presence of *E. coli* bacteria, all of the cytokines studied (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, Rantes, and gamma interferon [IFN-γ]) had more dense RT-PCR bands than when unstimulated PBMCs were used (data not shown). There were no significant differences between the nonpathogenic (EFC1 and FN414) and pathogenic (E2348/69 and H10407) *E. coli* strains. Similar results were obtained with formalin–heat-inactivated bacteria: there were no significant differences in cytokine expression between pathogenic and nonpathogenic strains of *E. coli* (data not shown). When French press bacterial lysates (50 μg/ml) were used to stimulate PBMCs, similar results were found except that lysates derived from EPEC strain E2348/69 did not induce the expression of IL-2 mRNA and induced only very low levels of IL-4 and IL-5 (data not shown).

Inhibition of lymphokine mRNA expression by lysates from EPEC. Next, studies were carried out to determine if the low levels of IL-2, IL-4, and IL-5 mRNA in cultures containing lysates from EPEC strain E2348/69 were due to a lack of stimulation or to inhibition of cytokine mRNA expression by activated PBMCs. As in the previous studies, 1.5×10^6 PBMCs were cultured with French press lysate (50 μg/ml) for an initial 2 h. After this preincubation period, PMA (20 μg/ml) and PWM (dilution, 1:100) were added to induce the expression of cytokine mRNA.

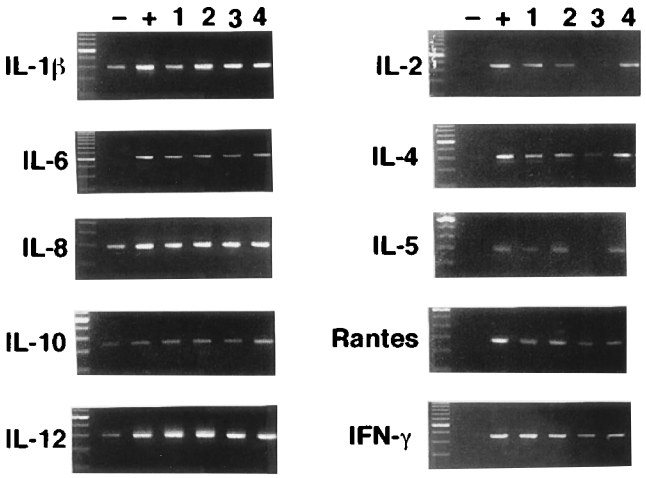

FIG. 1. Inhibition by bacterial lysates of lymphokine expression by mitogen-stimulated PBMCs. PBMCs (1.5×10^6) were cultured with bacterial lysates (50 μg/ml) from nonpathogenic *E. coli* strains EFC1 (lane 1) and FN414 (lane 2) and pathogenic *E. coli* strains EPEC E2348/69 (lane 3) and H10407 (lane 4) as for Fig. 2. After 2 h PMA-PWM was added (each at 20 ng/ml and a 1:100 dilution), and incubation was continued for an additional 6 h. In lane 3, IL-2 mRNA was absent and IL-4 and IL-5 mRNA were diminished when cells were preincubated with lysate from EPEC. Negative controls (−) consisted of PBMCs cultured with medium alone for 8 h. Positive controls (+) consisted of cells precultured for 2 h in medium alone and then for 6 h with PWM-PMA (see Materials and Methods). The left lane contains a 1-kb molecular weight ladder.

As shown in Fig. 1, RT-PCR bands for IL-2, IL-4, and IL-5 were either absent or very faint in cells pretreated with EPEC strain E2348/69 lysate (lane 3, right side), whereas cells pretreated with lysates from other bacterial strains expressed these lymphokines. IFN-γ and Rantes (Fig. 1, right side) and none of the monokines investigated (Fig. 1, left side) were affected by lysate from EPEC E2348/69, as all showed more dense bands upon stimulation with PMA-PWM. As shown in Fig. 2, the inhibitory effect of the EPEC lysate was dose dependent. Increasing amounts of EPEC E2348/69 French press lysates (1 μg/ml to 1 mg/ml) were added to 1.5×10^6 PBMCs. After an initial 2 h, PMA and PWM were added and RNA was isolated after a total of 8 h of culture. Complete inhibition of

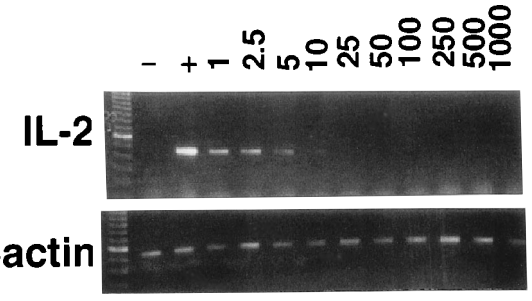

FIG. 2. Dose-dependent inhibition of IL-2 mRNA expression by lysate from EPEC 2348/69. PBMCs (1.5×10^6) were preincubated with increasing amounts of press lysate from *E. coli* 2348/69 (range, 1 to 1,000 μg/ml, as indicated above each lane). PWM-PMA was added after 2 h as for Fig. 3. Negative (−) and positive (+) controls were used as described in the legend to Fig. 1. Cytokine mRNA concentrations were determined by RT-PCR after a total of 8 h of culture. The left lane contains a 1-kb molecular weight ladder.

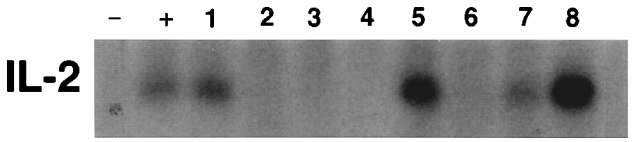

FIG. 3. Northern blot of IL-2 mRNA expression in mitogen-stimulated PBMCs after incubation with lysates from pathogenic *E. coli* strains. PBMCs (20×10^6) were preincubated with 250 μg of lysates per well from EPEC strains 0659/79 (lane 1), 2087/77 (lane 2), 2348/69 (lane 3), and 2430/78 (lane 4) and the enteroaggregative strain 17-2 (lane 5), EHEC strain EDL933 (lane 6), and enterotoxigenic strains H10407 (lane 7) and CFT325 (lane 8) (see Materials and Methods). After 2 h of culture with lysates, PMA-PWM (each at 20 ng/ml and a 1:100 dilution) was added. RNA was isolated after a total of 8 h of culture. Negative (−) and positive (+) controls were used as described in the legend to Fig. 1.

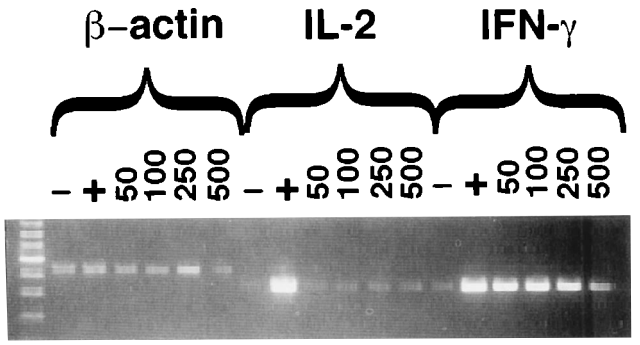

FIG. 4. Lysates from a plasmid-cured strain of EPEC inhibit mitogen-stimulated lymphokine mRNA expression. Lysates from EPEC JPN15, a plasmid-cured EPEC 2348/69 strain, were added to 1.5×10^6 PBMCs for 2 h; this was followed by an additional 6 h of culture with PMA-PWM. Negative (−) and positive (+) controls were used as described in the legend to Fig. 1. The left lane contains a 1-kb molecular weight ladder.

IL-2 mRNA expression was reproducibly observed with 25 μg of EPEC E2348/69 lysate per ml.

The inhibition of lymphokine mRNA expression was not due to cell toxicity. Cells remained viable after culture with lysates from EPEC strain E2348/69, as determined by a trypan blue exclusion test. In addition, β-actin mRNA expression was not affected. As indicated above, EPEC E2348/69 lysates inhibited expression of some, but not all, cytokine mRNAs tested. These results suggested that EPEC contains a factor or factors that inhibit lymphokine mRNA expression.

Cytokine mRNA inhibitory activity in different *E. coli* strains. Experiments were carried out to determine whether different pathogenic *E. coli* strains contain factors that inhibit cytokine mRNA expression. PBMCs were cultured in the presence of lysates from different bacteria, followed by PWM-PMA stimulation, and RNA was isolated after 8 h of culture. In these experiments, to validate the results obtained using RT-PCR, RNA was hybridized on Northern blots with a radioactive [^{32}P]CTP-labeled IL-2 DNA probe. As shown in Fig. 3, lysates from the EPEC strains E2348/69, 2087-77, and 2430-78 and the closely related enterohemorrhagic *E. coli* (EHEC) strain EDL933 inhibited IL-2 mRNA expression to levels that were undetectable. Lysates from the EPEC strain 0659-79, enteroaggregative strain 17-2, enterotoxigenic strain H10407 (a known heat-labile-enterotoxin-producing strain), and the uropathogenic strain CFT325 did not. These results indicate that the inhibitory factor is found in some, but not all, strains of EPEC and that it is present in another related *E. coli* strain, EHEC EDL933.

Inhibition of IL-2 protein expression by *E. coli* lysates. To investigate further whether the inhibition of IL-2 mRNA correlated with inhibition of IL-2 protein expression, culture supernatants from PBMCs were tested for IL-2 by ELISA (Table 3). Cells were incubated for a total of 24 h with French press lysate, and PMA-PWM was added after 2 h. After 24 h there was no detectable IL-2 protein in the supernatant of PBMCs that were treated with lysates from EPEC 2087-77, E2348/69, and 2430-78. IL-2 protein concentrations in the supernatants were not above background, confirming our results of complete absence of IL-2 mRNA (see above). As expected, the expression of IL-2 protein by PBMCs was not inhibited by lysates derived from EPEC 0659-79, although it was lower in comparison with the positive control.

TABLE 3. Lysates from EPEC strains inhibit IL-2 protein production by mitogen-stimulated PBMCs

Source of lysate	PWM-PMA	IL-2 (IU/ml)
None	None	0.0
None	+	29.8
EPEC strain 0659/79	+	12.1
EPEC strain 2087/77	+	0.0
EPEC strain 2348/69	+	0.2
EPEC strain 2430/78	+	0.0

PBMCs (1.0×10^6) were cultured for 2 h with culture medium or culture medium supplemented with bacterial lysates (see Materials and Methods), following which cells were cultured for a total of 24 h. Culture supernatants were tested for IL-2 by ELISA. Bacterial lysates did not have IL-2 reactivity or inhibit reactivity of IL-2 standards in the ELISA (data not shown).

Inhibitory activity in *E. coli* lysates is due to one or more proteins. Further studies were carried out to determine whether the inhibitory activity in lysates from EPEC E2348/69 was due to a protein. EPEC E2348/69 French press lysates were incubated with a solid-phase protease (Pierce, Rockford, Ill.) overnight or were heated to various temperatures for various periods of time. In these experiments, the inhibitory activity was absent in lysates treated with protease or heated to either 100 or 85°C for 10 min. Furthermore, the factor was retained in the bacterial lysate after dialysis with a 6,000- to 8,000-molecular-weight-cutoff membrane (data not shown).

To investigate whether the inhibitory activity observed with French press lysates from EPEC E2348/69 was due to arachidonic acid metabolites, PBMCs were initially incubated with increasing dosages of indomethacin (maximum dosage, 1 μg/ml [38]). After 2 h, PMA and PWM were added, and the cells were assayed for the expression for IL-2 mRNA by RT-PCR after 8 h of culture. IL-2 mRNA remained undetectable by PCR for any indomethacin dosage tested (data not shown), suggesting that the inhibitory activity was not due to an increase in prostaglandins.

Inhibitory activity of EPEC E2348/69 mutants. In parallel to experiments studying the activity of lysates derived from EPEC, we attempted to identify the genetic loci responsible for this activity. As shown in Fig. 4, lysates from the plasmid-cured EPEC strain 2348/69 JPN 15 (22) were able to inhibit the expression of IL-2 at the lowest concentration tested (50 μg/ml). IFN-γ mRNA expression was inhibited at higher concentrations of JPN 15 lysate. Thus, the inhibitory activity derived from EPEC 2348/69 does not depend on the presence of the EPEC plasmid and is therefore localized on the bacterial chromosome.

To further localize the inhibitory activity, a series of mutant strains were also tested. These strains carry insertion mutations in genes on the chromosome and plasmid that are responsible

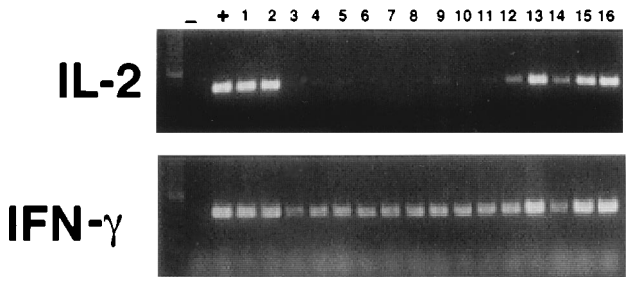

FIG. 5. Inhibitory activity of Tn*phoA* mutants of wild-type 2348/69. Lysates were prepared from EPEC having mutations that inactivate known pathogenicity factors. PBMCs (1.5×10^6) were incubated with lysates as for Fig. 3, followed by activation with PMA-PWM. All mutants tested had inhibitory activity similar to that of wild-type EPEC. Lanes: 1, EFC1; 2 to 5, EPEC strains 0659-79, 2087-77, 2430-78, and E2348/69, respectively; 6, 31-6-1; 7, CVD206; 8, UMD864; 9, 16-9-1; 10, 14-2-1; 11, 27-3-2; 12, 30-5-1; 13, 17-2; 14, EDL933; 15, H10407; 16, CFT325.

for adherence, attachment, and internalization of EPEC E2348/69 into epithelial cells (Table 1). As controls, EPEC strains 0659-79, 2087-77, E2348/69, and 2430-78 and strains 17-2, EDL933, H104O7, and CFT325 were included. As shown in Fig. 5, all of the deletion mutants exhibited inhibitory activity comparable to that of the wild-type EPECs 2087-77, E2348/69, and 2430-78 and EHEC EDL933. Thus, the inhibitory activity is not dependent on any previously characterized virulence loci.

Effect of different mechanisms of cell activation. In an attempt to identify the genetic loci responsible for the inhibitory effect on PBMCs, we screened an EPEC cosmid library in laboratory *E. coli* strain HB101, which does not have inhibitory activity (data not shown). As the inhibitory activity was present in nanomolar concentration ranges, it was possible to detect lymphokine inhibition by pooling and lysing, first whole plates (96 clones), then each row (12 clones), and finally individual clones. Three different clones were isolated from a total of 960 screened which suppressed lymphokine expression in lymphocytes, designated IV-8-A, IX-2-C, and IX-4-D. All further studies were carried out with IV-8-A, containing the smallest cosmid insert (22 kb).

We conducted experiments to determine whether the inhibitory effect of EPEC lysate on cytokine mRNA expression was dependent on the mechanism of cell activation (Fig. 6). PBMCs were cultured with lysates as described above, and different combinations of potent inducers of T-cell activation: PHA alone, the combinations of PMA plus PHA, PMA plus PWM, and OKT-3 alone. French press lysate was added that was obtained from the plasmid clone IV-8-A carrying a 22-kb genomic DNA fragment with the inhibitory activity. In the absence of lysate, each of the four activators induced expression of IL-2 and IL-5. Addition of lysate from the cosmid clone IV-8-A inhibited IL-2 and IL-5 but not β-actin or IL-1β mRNA expression in a dose-dependent fashion. Inhibition of IFN-γ was observed at higher concentrations of lysate (data not shown). Thus, the inhibitory effect of EPEC lysate does not depend on the mechanism of lymphocyte activation.

Effect of *E. coli* lysate on lymphocyte proliferation. Since IL-2 is a growth factor for lymphocyte proliferation, the effect of bacterial lysates on mitogen-stimulated lymphocyte proliferation was examined. Increasing concentrations of French press lysate derived from *E. coli* HB101 with and without the inhibitory cosmid clone were added to 2×10^5 PBMCs per microculture well. Proliferation of PBMCs was determined in triplicate after 3 days by [^{3}H]thymidine incorporation for 4 h.

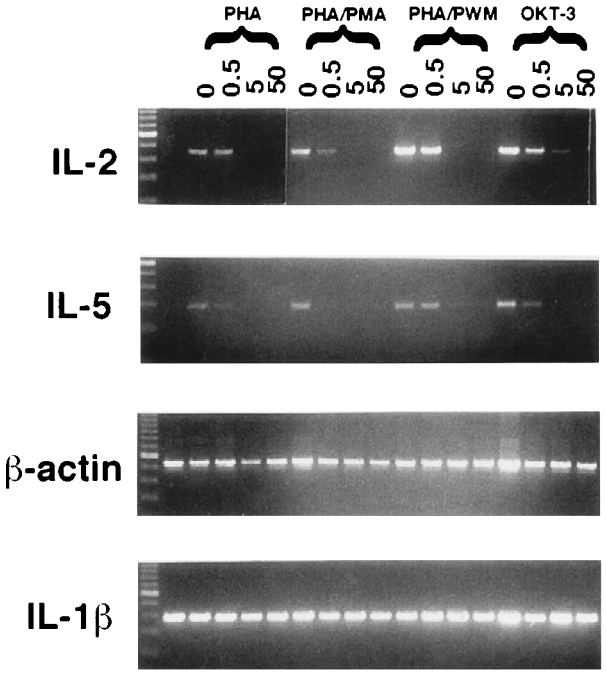

FIG. 6. Inhibitory effects of EPEC lysates with different stimuli for activation of PBMCs. PBMCs (1.5×10^6) were preincubated with lysates from an EPEC cosmid clone containing the inhibitory activity. After 2 h PHA alone, PHA plus PMA, PMA plus PWM, and OKT-3 alone were added for a total of 8 h of culture. Lanes indicate concentrations (micrograms per milliliter) of lysate added. The left lane contains a 1-kb molecular weight ladder.

As shown in Fig. 7, the lysate completely inhibited the proliferative response of PBMCs to PWM. A half-maximal suppression was seen with less than 1 μg of lysate of the HB101 clone IV-8-A per ml. Increasing dosages of HB101 without the cosmid DNA did not inhibit proliferation of PBMCs.

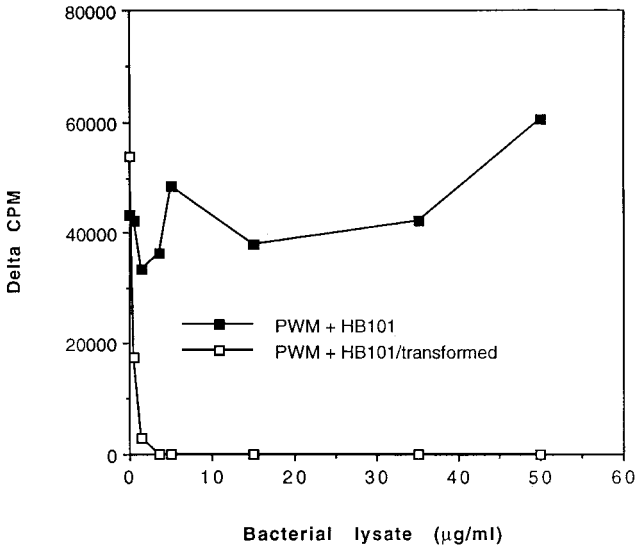

FIG. 7. Lysate from EPEC inhibits mitogen-stimulated proliferation of PBMCs. Increasing concentrations of lysates from the *E. coli* laboratory strain HB101 or from HB101 transformed with an EPEC cosmid clone were added to PBMCs. After 2 h, cells were washed and cultured with PWM. Proliferation ([^{3}H]thymidine incorporation) was determined on day 3 of culture. Values shown are mean counts per minute for triplicate cultures.

DISCUSSION

The results of this study show that lysates from certain strains of pathogenic *E. coli* can selectively inhibit mitogen-stimulated cytokine expression by human lymphocytes. Initially, in experiments using unfractionated, live bacteria, there were no apparent differences in the ability of nonpathogenic and pathogenic *E. coli* strains to stimulate cytokine mRNA expression as detected by PCR. The stimulatory factors which induce cytokine mRNA expression were not characterized in this study, but may include common determinants such as bacterial lipopolysaccharides that are potent stimuli for activation of the immune system. Surprisingly, when bacterial lysates were added to cultures of PBMCs, certain strains failed to induce expression of some lymphokine mRNAs. In further studies, it was shown that the absence of lymphokine mRNA expression was not due to lack of stimulatory activity but rather to the presence of a factor or factors that inhibit mitogen-stimulated lymphokine mRNA expression in lymphocytes. Interestingly, the factor does not inhibit expression of cytokines expressed predominantly in monocytes. The inhibitory activity was shown to be due to a protease-sensitive, heat-labile, nondialyzable, nontoxic factor that was present in some but not all EPEC strains and the EHEC strain EDL933. Furthermore, the inhibitory activity was independent of the mechanism of activation of PBMCs and was able to inhibit lymphocyte mRNA expression and proliferation. Finally, initial genetic characterization suggests that the factor is encoded by a previously undefined chromosomal gene that is unrelated to other known pathogenicity factors in EPEC. These observations have implications for the pathogenesis of *E. coli*-induced human disease, the role of the enteric flora in modulation of the immune system, and the pathogenesis of human inflammatory bowel disease.

EPEC organisms are a group of bacterial strains that share certain characteristics that distinguish them from other *E. coli* strains. They are a common cause of diarrhea, primarily among infants in developing countries, leading to widespread morbidity and mortality. Some of the pathogenic steps that appear to be important with this group of organisms include initial localized adherence to epithelial cells, followed by intimate attachment, which is associated with loss of microvilli and proliferation of filamentous actin beneath the site of attachment in the cell (9). These inducible pathogenic steps are facilitated by plasmid-encoded products for adherence and chromosomal genes for intimate attachment and effacement. At the present time there is no evidence that the pathogenic effects of EPEC are due to expression of toxins. It is of interest that in the present study, the factor that inhibited lymphokine mRNA expression was found in all (2087-77, E2348/69, and 2430-78) but one (0659-79) strain of EPEC. This may be attributable to the known diversity of EPEC strains (20). It was also of interest that although not present in enteroaggregative strain 17-2 or enterotoxigenic strain H10407 of *E. coli*, it was identified in the one strain of EHEC (EDL933) tested, which may be attributable to the known close evolutionary relationship between EPEC and EHEC strains (12). Studies of numerous laboratory strains available to us showed that the activity was present in a plasmid-cured strain and present in mutants, including those with mutations in the earlier defined *eaeA* gene. These findings suggest the possibility that the factor or factors that inhibit lymphokine expression may be unrelated to previously described virulence factors in EPEC. It is uncertain whether this newly identified activity is important in the pathogenesis of human EPEC, since the effect of infection on immune function is not known. Although the pathogenic effects of *E. coli* EDL933 have largely been attributed to the presence of a Shiga-like toxin (4, 36), the observation that this organism also contains a factor that could potentially be immunosuppressive raises that possibility that another pathogenic mechanism could play a role in this human infection.

An interesting aspect of the bacterial lysates studied was their differential effect on monokine and lymphokine expression. Although expression of certain lymphokine gene mRNAs was inhibited in mitogen-activated PBMCs (IL-2, IL-4, IL-5, and IFN-γ), cytokines expressed predominantly in monocytes were not altered, and in particular, the lysates contained factors that stimulated expression of these cytokines (including IL-1β, IL-6, IL-8, IL-10, IL-12, and Rantes). Since there appeared to be no differences between the abilities of nonpathogenic and pathogenic *E. coli* strains studied to induce these cytokine mRNAs, the stimulatory activity was not further characterized. However, it is well known that common factors such as lipopolysaccharides are potent inducers of monocyte activation and inflammation (4, 21). Bacterial lipopolysaccharides have long been thought to play an important role in maintenance of the normal mucosal immune system (22). In this regard, the absence of a normal flora and bacterial lipopolysaccharides has been suggested to account for the observation that germfree animals have a largely atrophic gastrointestinal immune system. Recolonization of the gastrointestinal tract is associated with the appearance of the large population of lymphocytes and monocytes normally present in this site. The results of the present study indicate no significant differences in the ability of EPEC to stimulate expression of inflammatory mediators in comparison with nonpathogenic *E. coli* strains. Therefore, in vivo when EPEC organisms are present in the gastrointestinal lumen or when they gain direct access to the mucosal immune system, such as through M cells (28), it would be expected that EPEC strains would have the same potential for activation of proinflammatory mechanisms as other *E. coli* strains. However, during prolonged infection with EPEC if products of EPEC gained direct access to the immune system, the results of the present study raise the possibility that inhibition of lymphocyte function might occur. Although there is no direct evidence concerning the existence of such a pathogenic mechanism, in malnourished infants already having some degree of immunodeficiency as a result of nutritional deficiency, the outcome could be significant with regard to further immunosuppression. Therefore, EPEC could play a dual role in the relentless downward spiral composed of diarrhea, malnutrition, and immunosuppression that culminates in a fatal outcome.

Another potential implication of the present study is suggested by recent observations of experimental animals having disrupted cytokine or T-cell-receptor genes. Sadlack et al. (32) have shown that mice lacking a functional II-2 gene spontaneously develop anti-colon antibodies and a severe form of hemorrhagic colitis. Furthermore, such animals maintained in a germfree environment have only minimal evidence of colonic abnormalities. In another model system, Mombaerts et al. (25) found that T-cell-receptor-deficient mice also develop chronic colonic inflammation. Finally, Kühn et al. (19) found that IL-10-deficient mice also develop gastrointestinal inflammation, but in this case the disease is enterocolitis. As in the case of IL-2-deficient mice, a role for the enteric bacteria is suggested by mild disease in animals kept in pathogen-free conditions. Although the details of the pathogenic mechanisms involved in these different models are yet to be determined and may be complex, the general implication of these studies is that inhibition of the normally finely regulated gastrointestinal mucosal immune system in the presence of enteric bacteria in the gas-

trointestinal lumen may result in significant gastrointestinal inflammatory disease. The results of the present study therefore are of particular interest in that they suggest a possible mechanism by which enteric bacteria may produce an outcome similar to that observed in genetically altered mice. Products of EPEC or EHEC on the one hand may markedly stimulate an inflammatory response, with liberation of cytokines, while at the same time inhibiting expression of lymphokines. These general effects could be entirely unrelated to any antigen-specific immune response. In conclusion, the results of the present study suggest a potential new mechanism by which products of enteric bacteria could modify lymphocyte immune function in the gastrointestinal tract.

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