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The Diarrheal Response of Humans to Some Classic Serotypes of Enteropathogenic *Escherichia coli* is Dependent on a Plasmid Encoding an Enteroadhesiveness Factor

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Isolates of the most common O serogroups of enteropathogenic *Escherichia coli* (EPEC) associated with infant diarrhea (designated class I) adhere to Hep-2 cells; the genes for this adhesin, termed EPEC adherence factor (EAF), are located on plasmids 50–70 MDa in size. Volunteers ingested 10^{10} organisms of an O127:H6 Hep-2–adhesive class I strain (E2348/69) or its plasmid-minus, nonadhesive derivative. Diarrhea occurred in nine of 10 volunteers who ingested the parent strain (mean, 1,178 ml) but in only two of nine who took the plasmid-minus variant (mean, 433 ml; $P < .006$). All volunteers ill from strain E2348/69 mounted serum IgA and IgG responses to a 94-kDa plasmid-associated outer membrane protein of E2348/69; this protein was found in other class I EPEC but not in enterotoxigenic or meningitic strains. The 50–70-MDa EAF plasmid seems necessary for full expression of pathogenicity in EPEC that exhibit Hep-2 adhesiveness. EPEC isolates of certain other, less common, O serogroups (O44, O86, and O114) are rarely Hep-2 adhesive. These EPEC, designated class II, possess distinct 50–70 MDa plasmids lacking EAF genes. Diarrhea was caused by 10^8 or 10^{10} organisms of an O114:H2 class II EPEC strain (mean, 1,156 ml) in six of 11 volunteers. This result confirmed that class II EPEC are pathogenic by a mechanism not involving Hep-2 adhesiveness.

Enteropathogenic *Escherichia coli* (EPEC) refers to certain serotypes of *E. coli* that were first incriminated in epidemiological studies in the 1940s and 1950s as causes of epidemic and sporadic infant diarrhea [1–5]. By the late 1950s, the standard technique for detecting these pathogens involved identifying them by agglutination with appropriate antisera [6–8]. Although no biochemical, microbiological or animal-model assays at that time could demonstrate an inherent pathogenicity vis-à-vis other *E. coli*, experimental challenge studies in the early 1950s in the United States, England, and Japan confirmed that strains of serogroups O55, O111, and O127, isolated from infants with gastroenteritis, caused diarrhea when fed to volunteers [9–14]. Also at this time, Ewing et al. [6, 8, 15] in the United States and Taylor [7] in England noted that among all the EPEC, certain O serogroups, including O26, O55, O111, O119, O127, and O128, were particularly frequent and epidemiologically well-incriminated in association with diarrhea, whereas isolates of other EPEC O serogroups, such as O44, O86, and O114, were less common. Serogroup O142 had not yet been recognized as an EPEC at the time of those publications.

Interest in the pathogenesis of EPEC diarrhea was markedly renewed in 1978, when it was shown that EPEC strains of serogroups O127 and O142 caused a notable diarrheal illness in young adult volunteers

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Informed consent was obtained from participants in this clinical research study, which followed the guidelines of the Department of Health and Human Services. The protocol was approved by the Human Volunteer Research Committee at the University of Maryland and the Clinical Review Sub-Panel of the National Institute of Allergy and Infectious Diseases.

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[16], although these strains did not elaborate heat-labile (LT) or heat-stable (ST) enterotoxins and did not manifest shigella-like invasiveness for epithelial cells. These investigators concluded that EPEC cause diarrhea by other pathogenetic mechanisms. Shortly thereafter, Cravioto et al. [17] found that 80% of the EPEC strains that they examined adhered to Hep-2 cells in tissue culture in the presence of D-mannose, a property found to be uncommon in other *E. coli*. Examination of the ultrastructure of EPEC infection in intestinal biopsies of ill infants [18, 19] and in animal models [20, 21] has revealed a distinct histopathologic lesion in which the EPEC intimately adhere to enterocytes, an occurrence resulting in dissolution of microvilli and cupping of the enterocyte outer membrane around the bacteria. So far, this lesion has been identified in infections due to EPEC of serogroups O26, O55, O111, O114, O119, O125, O127, and O142.

Baldini et al. [22] noted that 31 of 32 EPEC strains possessed a plasmid 50–70 MDa in size. Working with E2348/69, an O127:H6 strain that causes diarrhea in volunteers, these workers found that the genes encoding Hep-2 adhesiveness and the ability to adhere to the intestinal mucosa of colostrum-deprived piglets are encoded on a 60-MDa plasmid. The term EPEC *adherence factor* (EAF) has been suggested to refer to the plasmid-mediated adhesin that confers Hep-2 adherence.

Nataro et al. [23, 24] and Baldini et al. [25] have isolated a one-kilobase pair (Kb) fragment from the 60-MDa plasmid of E2348/69 that has proved to be a highly sensitive and specific DNA hybridization probe for detecting EPEC strains that exhibit Hep-2 adhesiveness. In field studies in Peru with this DNA gene probe, these workers noted that Hep-2 adhesiveness was more frequently found among EPEC serogroups considered to be the most important causes of epidemic and sporadic EPEC diarrhea worldwide and that this trait correlated with the pathogenicity of these strains. In contrast, EPEC of the serogroups O44, O86, and O114, rarely found causing outbreaks and of generally lesser significance in sporadic diarrhea, were associated with diarrhea though they did not carry genes for EAF. In light of these observations, Nataro suggested the following designations: class I for serogroups O55, O111, O119, O127, O128, and O142; and class II for serogroups O44, O86, and O114.

Volunteer studies with representative class I and II EPEC strains were carried out to determine whether the 60-MDa plasmid of E2348/69 (O127:H6) affects pathogenicity and whether an O114:H2 class II EPEC is capable of causing diarrhea, although lacking the genes for Hep-2 adhesiveness.

Subjects and Methods

Bacterial strains. E. coli strain E2348/69 (O127:H6), isolated during an outbreak of infant diarrhea in Taunton, England [26], was provided by Bernard Rowe (Division of Enteric Pathogens, Central Public Health Laboratory, London). Strain E2348/69 possesses plasmids of 60 and 5 MDa [22], adheres to and forms microcolonies on Hep-2 cells [22], attaches to the intestinal mucosa of colostrum-deprived piglets [21], and hybridizes with a gene probe that detects Hep-2–adhesion genes [23, 24]. Strain MAR 20 is a derivative of E2348/69 cured of the 60-MDa plasmid, as described previously [22], that cannot adhere to Hep-2 cells or piglet intestinal mucosa and is probe negative. *E. coli* E128010 (O114:H2) was isolated from an infant with sporadic diarrhea in Bangladesh [27]. This strain possesses two plasmids, of 50 and 60 MDa, but does not adhere to Hep-2 cells and is probe negative. As previously reported, the above strains do not carry genes for ST or LT production, do not hemagglutinate human or bovine red blood cells, and are not invasive in the guinea pig keratoconjunctivitis test [16, 27, 28]. Other pertinent microbiological and biologic properties of these strains are summarized in table 1.

Strains E2348/69, MAR 20, and E128010 were examined for their ability to produce Shiga-like toxin by the HeLa-cell cytotoxicity method of O'Brien et al. [29]. Extracts of strains that were cytotoxic for HeLa cells were reacted with specific Shiga antitoxin to determine if the cytotoxicity could be neutralized.

Nine other *E. coli* strains were selected for examination of their outer membrane proteins. These included the following: enterotoxigenic *E. coli* strains B_2C (O6:H16), H10407 (O78:H11), M424C1 (O6:H16), and B7A (O148:H28) [30, 31]; strains RS188 (O7:K1:H−) and RS408 (O75:K1), associated with neonatal meningitis and provided by Richard Silver (Bureau of Biologics, Bethesda, Md); EPEC strain E851/71 (O142:H6) [16]; and EPEC strains 0659/79 (O119:H6) and 2340/78 (O111a,b:H−), provided by I. Kaye Wachsmuth (Centers for Disease Control, Atlanta).

Volunteers and study design. Volunteers were

Table 1. Biologic characteristics of three enteropathogenic strains of *E. coli* fed to healthy adult volunteers.

Strain	Origin	Serotype	Adhesion to Hep-2 cells with microcolony formation	Plasmids (MDa)	DNA hybridization probe for Hep-2-adhesive (EAF) genes
E2348/69	Infant nursery diarrhea outbreak, Taunton, England	O127:H6	+	60 5	+
MAR 20	Laboratory-derived, plasmid-cured variant of E2348/69	O127:H6	–	5	–
E128010	Sporadic infant diarrhea, Bangladesh	O114:H2	–	60 50	–

college students and other healthy adults aged 18–35 years from the Baltimore community; they were admitted to the 22-bed Isolation Ward of the Center for Vaccine Development for a period of 12 days. Methods of recruitment, medical screening, and clinical supervision have been previously described [16, 32]. The studies were explained in detail to the volunteers and signed, witnessed consent was obtained. In order to ensure the informed nature of consent, volunteers were required to pass a written examination containing multiple choice and true-false questions on all aspects of the study, including risks, benefits, procedures, and microbiology [16, 32].

In the first study, 19 volunteers were randomly chosen to receive 10^{10} *E. coli* strain E2348/69 or its plasmid-minus derivative, *E. coli* strain MAR 20. Neither the clinical observers nor the volunteers were aware of which strain they received.

In the second study, a total of 11 volunteers received 10^8 or 10^{10} *E. coli* strain E128010. The inocula for bacterial challenge were prepared as previously described [16] and were fed to fasting volunteers with 2.0 g $NaHCO_3$ in 150 ml of water.

The volunteers were closely observed for a period of 96 hr after challenge, during which time all stools were collected, examined, graded, and cultured. Rectal swabs were obtained if a daily stool was not passed. Diarrhea was defined as two or more loose stools within 48 hr totalling at least 200 ml in volume or a single loose stool of $\geqslant$300 ml in volume [32]. After 96 hr of observation, all volunteers were given oral neomycin (500 mg every 6 hr) or colistimethate (300 mg every 8 hr) for five days to eradicate the challenge strains.

Bacteriology. Stools and rectal swabs were cultured on Levine's eosin methylene blue (EMB) agar with and without nalidixic acid, 50 μg/ml (all challenge strains were nalidixic-acid resistant). Quantitative stool cultures were performed as previously described [16].

Probe hybridization. From stool cultures of volunteers who ingested E2348/69, lactose-positive colonies from the EMB plate containing nalidixic acid were agglutinated with OK antiserum prepared to E2348/69. After challenge, five agglutinating colonies per volunteer per day were saved to be tested with the DNA probe that detects the Hep-2-adherence genes. The colony hybridization technique of Moseley et al. [33] was followed with stringent conditions (50% formamide). In this way, the stability of the 60-MDa plasmid of E2348/69 in human intestinal infection was assessed.

Plasmid extraction. Plasmid extraction was performed by the rapid alkaline-extraction procedure of Birnboim and Doly [34], and plasmid profiles were visualized after electrophoresis through 0.7% agarose gels.

Serology. Sera collected before and 10, 21, and 28 days after challenge were examined for antibody to O127 or O114 O antigens by using the passive HA technique as previously described [16, 35]. The O127 lipopolysaccharide was a phenol-water preparation (List Biologicals, Campbell, Calif) [36]. The O114 antigen was obtained by heat-alkaline treatment [37].

Outer membrane preparations. The *E. coli* strains were grown overnight in Pen Assay broth (Difco, Detroit) at 37 C, and outer membrane preparations (OMPs) were made by the method of Achtman et al. [38]. The protein content of the OMPs was measured by the technique of Markwell [39]. Samples of OMP (containing 30 μg of protein) were electrophoresed in 11% polyacrylamide gel by the technique of Lugtenberg et al. [40] in the presence of SDS and the electrophorogram stained with coomassie blue.

Immunoblotting. Replicate 200-μg samples of

E2348/69 OMP were subjected to SDS-PAGE. After electrophoresis, the proteins were transferred from polyacrylamide gel to nitrocellulose paper by vacuum blotting for 2 hr by the procedure of Peferoen et al. [41], and the paper was cut into strips. The unabsorbed sites on the strips were blocked by incubation for 1 hr at room temperature in PBS containing 0.5% polyoxylenesorbitan monolaurate (Tween-20; Fisher Chemical, Pittsburgh). The strips were then incubated overnight in 2 ml of pre- (day 0) or postchallenge (day 28) human serum (challenged with E2348/69 or MAR 20) diluted 1:100 in PBS containing 0.5% Tween-20. The strips were washed three times (20 min each) in buffer before adding alkaline phosphatase–conjugated goat antibody to human IgA or antibody to IgG (Jackson Immunoresearch, Hamburg, FRG) for 2 hr at room temperature. Following three 10-min washes in PBS/0.5% Tween-20, the strips were exposed to 5-bromo-4-chloroindoxyl phosphate (Sigma, St. Louis) substrate in the presence of nitroblue tetrazolium (Sigma), according to the method of Blake et al. [42], for 10 min at 37 C to develop a colorimetric reaction. In later experiments, OMPs of the other strains were submitted to PAGE, vacuum blotted onto nitrocellulose filters, reacted with day-28 convalescent serum from a volunteer who developed diarrhea with strain E2348/69, and treated with conjugated antibody to IgA followed by substrate.

Results

Class I EPEC. Shiga toxin. Both strain E2348/69 and its plasmid-minus derivative MAR 20 produced trace amounts of a Shiga-like toxin that was neutralized by specific Shiga antitoxin. Trace amounts of Shiga-like toxin were defined as ≤200 50% HeLa-cell cytotoxic doses per mg of cell-lysate protein.

Clinical. Diarrhea occurred in nine (90%) of 10 volunteers who received strain E2348/69 but in only two (22%) of nine who ingested the plasmid-minus derivative, MAR 20 ($P < .006$; table 2). The two ill volunteers who received the plasmid-minus derivative had milder diarrhea (mean stool volume, 433 ml) than did those who ingested the parent strain (mean stool volume, 1,178 ml). Most of the volunteers who ingested strain E2348/69 developed anorexia, malaise, and abdominal gurgling, whereas these symptoms were not seen in the two volunteers who developed mild diarrhea after ingestion of MAR 20.

Bacteriology. All volunteers became colonized by the organism they ingested, and the geometric mean level of fecal excretion was similar in both groups (7.3×10^7 E2348/g and 2.6×10^8 MAR 20/g).

Isolates of E2348/69 from stool cultures of volunteers who ingested that strain were tested for the presence of the Hep-2–adhesive genes by means of a gene probe. Of 174 E2348/69 isolates tested, only 57 (33%) were positive with the probe. Sixty of the 174 isolates were randomly selected for plasmid analysis. All the probe-negative isolates also lacked the 60-MDa plasmid; in contrast, all probe-positive isolates possessed the plasmid. The proportion of plasmid-minus, Hep-2–negative isolates was similar on all postchallenge days.

The possibility that spontaneous curing of the plasmid occurred in the act of preparation of the challenge inoculum was examined by a reconstruction experiment, in which the inoculum-preparation procedure was repeated and the organisms probed

Table 2. Clinical response of volunteers after ingestion of an O127:H6 EPEC (class I) strain with and without its 60-MDa plasmid or of an O114:H2, class II EPEC.

Strain ingested	Dose	Diarrhea attack rate*	Mean diarrheal stool volume (range)	Positive stool cultures*
E2348/69 (O127:H6, with plasmid)	10^{10}	9/10†	1,178 ml (207–3,224)	10/10
MAR 20 (O127:H6, without plasmid)	10^{10}	2/9†	433 ml (269–596)	9/9
E128010 (O114:H2)	10^8	3/6	1,090 ml (328–2,045)	6/6
	10^{10}	3/5	1,225 ml (390–1,785)	5/5

* No. positive/no. of volunteers challenged.
† $P < .006$ by two-tailed Fisher's exact test.

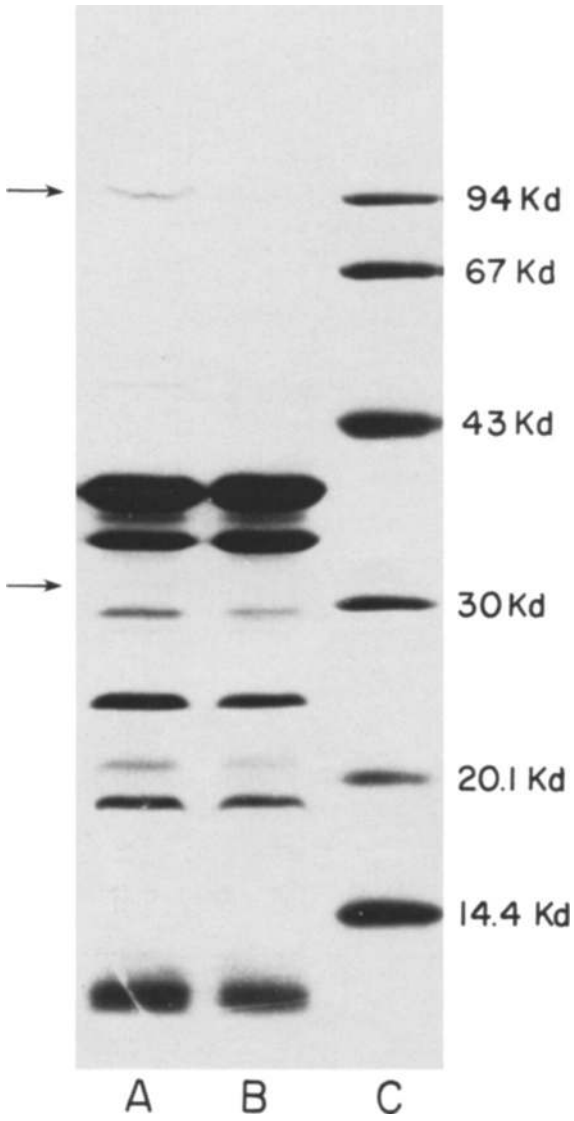

Figure 1. SDS-PAGE of outer membrane preparations (OMP) of *E. coli* strain E2348/69 (*lane A*) and its plasmid-minus derivative, MAR 20 (*lane B*). Molecular weight standards are in lane C (Low Molecular Weight Standards Kit; Pharmacia, Uppsala, Sweden). The upper arrow points to a prominent doublet protein band. The lower arrow points to a faint protein band.

after plating on Luria-agar (L-agar). The possibility that spontaneous cure occurred during the coproculture procedure was also examined by passing a fresh inoculum twice on EMB agar and twice on L-agar to mimic the clinical microbiological procedure and then performing the DNA hybridization. In both these instances, the prevalence of plasmid-minus isolates of E2348/69 was <1% of all colonies tested. Thus, the high prevalence (67%) of plasmid-minus colonies isolated from stool cultures of volunteers implies that plasmid curing occurred in vivo, in the human intestine.

Serology. The rate of seroconversion (≥fourfold rise) to O127 antigen was similar in volunteers who ingested E2348/69 (eight of 10) and MAR 20 (eight of nine). The peak geometric mean titer after challenge among those who exhibited seroconversions was 49 in the E2348/69 recipients and 41 in those who ingested MAR 20.

Outer membrane profile. Analysis of the OMP of *E. coli* strains E2348/69 and MAR 20 (figure 1) reveals a 94-kDa protein and a minor 31-kDa protein that are absent in the plasmid-minus derivative, MAR 20.

Immunoblotting. Prechallenge (day 0) and postchallenge (day 28) sera from volunteers who ingested either *E. coli* strain E2348/69 or its plasmid-minus derivative MAR 20, were tested in an immunoblotting procedure for the presence of specific IgA and IgG antibodies to E2348/69 OMP antigens.

Results with the conjugated antibody to IgA shown in figure 2A clearly demonstrate that volunteers challenged with the plasmid-containing parent strain, E2348/69, mounted a notable immune response to the 94-kDa plasmid-associated protein. One of 10 recipients of strain E2348/69 (volunteer 4) had detectable antibody to this protein in the prechallenge specimen. In the convalescent (day 28) sera, however, all 10 recipients of E2348/69 showed antibody to the 94-KDa protein, a result demonstrating that all nine seronegative volunteers seroconverted. It is of interest that the one volunteer who failed to develop diarrhea after ingestion of E2348/69 (table 2) was the individual in this group who had detectable antibody before challenge. One individual who ingested MAR 20 (volunteer 1, figure 2B) had detectable antibody in both pre- and postchallenge specimens; no volunteers in this group manifested seroconversions in antibody to the 94-kDa protein. Identical results were obtained when levels of IgG antibody were measured with conjugated antibody to IgG (data not shown). An immune response to the 31-kDa plasmid-encoded protein was not detected.

In figure 3, the day-28 serum of one E2348/69 volunteer who seroconverted is shown tested against E2348/69 OMP directly and after adsorption with MAR 20 or E2348/69 bacteria. The unadsorbed serum shows a strong antibody reaction to the 94-kDa

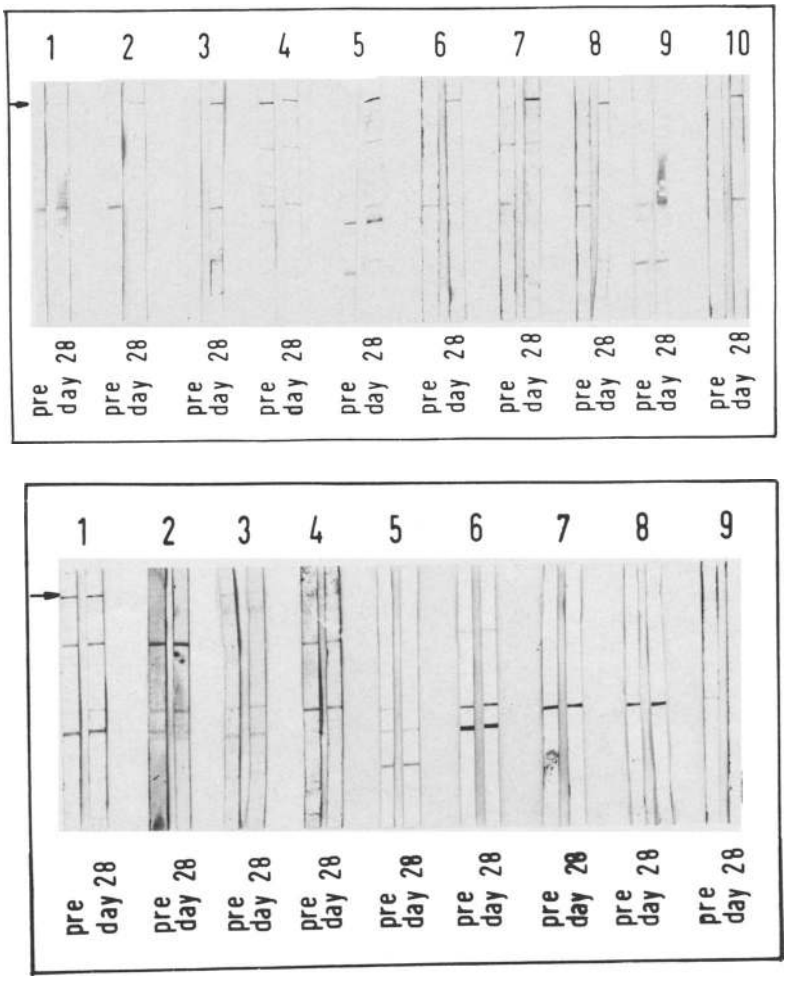

Figure 2. (*Top*) Immunoblot of sera of volunteers who ingested *E. coli* strain E2348/69 tested against strain E2348/69 OMP. The arrow points to the 94-kDa plasmid-associated protein. (*Bottom*) Immunoblot of sera of volunteers who ingested *E. coli* strain MAR 20 tested against strain E2348/69 OMP. The arrow points to the 94-kDa plasmid-associated protein.

protein and evidence of lesser amounts of antibody combining with other proteins (figure 3, lane A). Adsorption with E2348/69 cells removes virtually all antibody (figure 3, lane B). In contrast, adsorption with MAR 20 removes antibody to the other OMP proteins but leaves antibody to the 94-kDa protein (figure 3, lane C).

In view of the notable immune response demonstrated to the presumably plasmid-associated, 94-kDa protein, OMPs of 10 other selected strains of *E. coli* were examined for the presence of the 94-kDa protein in immunoblots by using day-28 convalescent sera from a patient who ingested E2348/69 as an immunologic probe. Results are shown in table 3. The four enterotoxigenic *E. coli* and two meningitis strains lacked the 94-kDa protein. In contrast, the O119, O111, and O142 EPEC strains, all of which are Hep-2 adhesive and positive with the DNA probe, possessed a protein of ~94 kDa that reacted with the convalescent antiserum. *E. coli* E128010, an O114 class II EPEC strain (see below) that is negative with the EAF gene probe and does not adhere to Hep-2 cells, was negative for the 94-kDa protein.

Class II EPEC. The O114:H2 EPEC strain (E128010) that was fed to volunteers caused unequivocal diarrheal illness in 50%–60%. The clinical and bacteriologic results are summarized in table 2. Like strain E2348/69, E128010 was found to produce trace levels of Shiga-like toxin. Nine of 11 recipients of E128010 manifested serological rises in titer to the O114 antigen.

Discussion

Plasmids encode critical virulence properties for enterotoxigenic [43–45], enteroinvasive [46], and enterohemorrhagic [47] *E. coli* that cause diarrheal disease [27]. It is now obvious that the 50–70-MDa

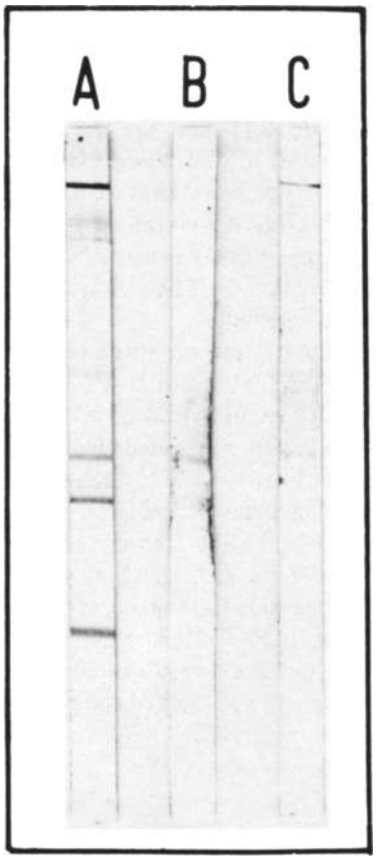

Figure 3. The day-28 postchallenge serum from a volunteer who ingested *E. coli* strain E2348/69 and seroconverted to the 94-kDa protein is shown tested against strain E2348/69 OMP. The serum was tested unadsorbed (*lane A*) and after adsorption with E2348/69 (*lane B*) or MAR 20 (*lane C*) bacteria.

plasmid that is present in class I EPEC also encodes genes that greatly affect the pathogenicity of such strains in humans. Both of the O127:H6 strains that were fed to volunteers had smooth lipopolysaccaride O antigens and elaborated similar levels of Shiga-like toxin. Fecal excretion was the same for both strains, as was the frequency and magnitude of the serum O-antibody response. However, the 60-MDa plasmid apparently encodes or regulates an adhesin that is not expressed in the absence of the plasmid. OMPs of E2348/69 and MAR 20 were examined because the observations of Scotland et al. [48] suggest that nonfimbrial adhesins are involved in attachment of EPEC to Hep-2 cells. Our studies revealed a 94-kDa outer membrane protein produced by E2348/69 but not by MAR 20, to which volunteers who ingested E2348/69 mounted an IgA and IgG immune response in serum (figures 1-3). This 94-kDa protein was absent in four enterotoxigenic *E. coli* strains and in two strains from patients with neonatal meningitis. In contrast, three Hep-2-adhesive, EAF gene probe-positive EPEC strains of serogroups O111, O142, and O119 were found to possess the 94-kDa outer membrane protein. These preliminary data suggest that this protein may play a role in the pathogenesis of diarrhea due to EPEC strains that cause localized adhesion on Hep-2 cells and are positive with the EAF gene probe. Studies are underway to investigate this association in a larger and more varied series of *E. coli* strains. It is of further interest that the one volunteer among the group of 10 who did not develop diarrhea when fed *E. coli* strain E2348/69 was the one individual in the group who, before challenge, had antibody to the 94 kDa

Table 3. Occurrence of a 94-kDa protein in outer membrane preparations of *E. coli* strains from various clinical sources, detected in immunoblots.

Class of *E. coli*	Serotype	Source	94-kDa outer membrane protein detected with immunologic probe
Enterotoxigenic			
B_2C	O6:H16	travelers' diarrhea	–
H10407	O78:H11	travelers' diarrhea	–
M424C1	O6:H16	travelers' diarrhea	–
B7A	O148:H28	travelers' diarrhea	–
K1 meningitic			
RS188	O7:K1:H–	neonatal meningitis	–
RS408	O75:K71	neonatal meningitis	–
EPEC			
0659/78	O119:H6*	infant diarrhea	+
2430/78	O111a,b:H–*	infant diarrhea	+
E851/71	O142:H6*	infant diarrhea	+†
E128010	O114:H2	infant diarrhea	–

* Serotypes form microcolonies on Hep-2 cells and are positive with the EAF gene probe.

† The outer membrane protein that reacted with the immunologic probe was 96 kDa.

protein. Although this observation involves only one patient, it should encourage further studies to assess the role of antibody to the 94-kDa protein in mediating protection and to investigate the protein as a potentially important antigen in development of future vaccines against class I EPEC.

Because the Hep-2–adherence plasmid is moderately large, there are ample segments of cryptic DNA that conceivably could encode other, currently unrecognized, virulence factors. We are currently analyzing the protein products of plasmid derivatives that are specifically inactivated in the Hep-2–adherence genes. A future volunteer study with the O127:H6 strain, containing the inactivated Hep-2 genes will definitively elucidate the role of these genes.

A surprise finding was that although the EAF plasmid is extremely stable in vitro, considerable spontaneous cure was observed after intestinal passage. It should be noted that this plasmid loss occurred after infection of adults, a group not considered naturally at high risk for this pathogen, and was observed for only one strain [1]. Plasmid loss in the intestine of young infants may be much less frequent. These data may provide a clue to a better understanding of the epidemiology of EPEC. For example, when identified by serotyping, the difference in rate of isolation of EPEC from infants with diarrhea vs. healthy controls is highly significant in infants less than six months of age, whereas the rates of isolation are similar in infants more than six months of age [1, 49]. On the basis of observations in the volunteer studies described herein, one would want in future epidemiological studies to ascertain whether the fecal isolates from class I EPEC from cases and controls of various ages possess EAF. It is conceivable that although the isolation of EAF-positive EPEC is high in very young infants with diarrhea, older children and healthy controls may more often be colonized with nonpathogenic, plasmid-minus variants, like our volunteers who were colonized with MAR 20. The observation of in vivo plasmid loss in volunteers also provides a note of caution to investigators examining EPEC strains from sporadic cases of diarrhea or from outbreaks. Multiple isolates from each individual must be examined before concluding that the implicated EPEC strain lacks the EAF plasmid. Epidemiological studies are underway to test these hypotheses.

Strain E128010, an O114:H2 class II EPEC that contains two large (50- and 60-MDa) plasmids but is Hep-2 negative and probe negative, nevertheless caused diarrhea of a severity equal to that in volunteers who ingested E2348/69 (table 3). This shows that EPEC strains of the serogroups recognized by Ewing et al. [6, 8, 15] and Taylor [7] as being less-important causes of diarrhea (in comparison with class I EPEC), are nevertheless clearly diarrheagenic pathogens. Studies are commencing to investigate whether the presumably distinct 50–70-MDa plasmids found in class II EPEC, such as E128010, play a role in pathogenicity.

Results of the volunteer studies corroborate the epidemiological findings of Nataro et al. [24] who, on the basis of examination of a large number of isolates from Peru, concluded that EAF is important for the pathogenicity of O serogroups designated class I (which includes O55, O111, O119, O127, O128, and O142), and that EPEC strains of class II (which contains the less common O44, O86, and O114 serogroups) are also pathogenic, even though they typically lack EAF. It is hoped that the preliminary studies described herein will stimulate other investigators to undertake studies of the pathogenicity of class I and class II EPEC.

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