Decreased Adherence of Enterohemorrhagic Escherichia coli to HEp-2 Cells in the Presence of Antibodies That Recognize the C-Terminal Region of Intimin

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Antiserum raised against intimin from enterohemorrhagic Escherichia coli (EHEC) O157:H7 strain 86-24 has been shown previously by our laboratory to inhibit adherence of this strain to HEp-2 cells. In the present study, we sought to identify the region(s) of intimin important for the effect of anti-intimin antisera on EHEC adherence and to determine whether antisera raised against intimin from an O157:H7 strain could reduce adherence of other strains. Compared to preimmune serum controls, polyclonal sera raised against the histidine-tagged intimin protein RIHisEae (intiminO157) or against His-tagged C-terminal fragments of intimin from strain 86-24 reduced adherence of this strain. Furthermore, an antibody fraction purified from the anti-RIHisEae serum that contained antibodies to the C-terminal third of intimin, the putative receptor-binding domain, also reduced adherence of strain 86-24, but a purified fraction containing antibodies to the N-terminal two-thirds of intimin did not inhibit adherence. The polyclonal anti-intiminO157 serum raised against RIHisEae inhibited, to different degrees, the adherence of another O157:H7 strain, an EHEC O55:H7 strain, one of two independent EHEC O111:NM isolates tested, and one of two EHEC O26:H11 strains tested. Adherence of the other O26:H11 and O111:NM strains and an EPEC O127:H6 strain was not reduced. Finally, immunoblot analysis indicated a correlation between the antigenic divergence in the C-terminal third of intimins from different strains and the capacity of anti-intiminO157 antiserum to reduce adherence of heterologous strains. Taken together, these data suggest that intiminO157 could be used as an immunogen to elicit adherence-blocking antibodies against O157:H7 strains and closely-related EHEC.

Infection of humans with enterohemorrhagic Escherichia coli (EHEC), such as the prototype E. coli O157:H7, can lead to diarrhea, hemorrhagic colitis, and, in approximately 5 to 15% of infected children, the hemolytic-uremic syndrome (HUS) (reviewed recently in references 23, 50, 51, and 53). EHEC is a subset of Shiga toxin-producing E. coli (STEC) that is characterized by Shiga toxin production, the presence of a 90-kb plasmid, and the capacity to produce attaching and effacing (A/E) lesions on epithelial cells in culture and in the intestines of experimentally inoculated animals (37). A/E histopathology results from intimate attachment of the bacteria to epithelial cells, effacement of the microvilli, and rearrangement of the host cell actin cytoskeleton (33, 49). The factors responsible for this attachment and associated events in the host cell are encoded in a pathogenicity island, the locus of enterocyte effacement (33), the C-terminal portion of the eae gene (67). Adherence of EHEC O157:H7 to human epithelial cells in vitro and colonization of experimentally infected animals require the function of the adhesin intimin, an outer membrane protein of approximately 94 to 97 kDa encoded by the eae gene (16, 46, 64). The eae gene was originally identified as essential for A/E lesion formation by enteropathogenic E. coli (EPEC), a related diarrhea pathogen that forms A/E lesions but does not produce Shiga toxins (30, 37). The importance of intimin for full virulence of EPEC was demonstrated in a study of infection by this organism of volunteers (14); the severity of Shiga toxin-mediated HUS has precluded human experimental challenge with EHEC. The sequences of intimin proteins from different strains of EPEC and EHEC and from several animal pathogens show a pattern of strong conservation in the central and N-terminal portions and more divergence in the C-terminal region (1–3, 31, 45). The C-terminal region of intimin has been shown to be critical for interaction with the human cell (13, 18, 19, 27, 32, 40).

Intimin is immunogenic in humans. Anti-intimin antibodies have been detected in milk and colostrum, in sera from individuals with EPEC infection, and in sera from HUS patients infected with STEC (29, 39, 43, 44, 47, 50, 65). The development of a multivalent anti-EHEC vaccine that would include Shiga toxins and intimin has been proposed by our laboratory and others (7, 12, 31). The inclusion of intimin in such a vaccine is based on the hypothesis that a sufficient titer of anti-intimin antibodies may reduce or inhibit colonization. Since intimin is an outer membrane protein, regions of the protein may be accessible for antibody binding to interfere with
TABLE 1. EHEC and EPEC strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype (EHEC or EPEC)</th>
<th>Intimin subtypea</th>
<th>Yr of isolation or date received at CDCb (reference), source</th>
</tr>
</thead>
<tbody>
<tr>
<td>O157:H7</td>
<td>EHEC</td>
<td>γ</td>
<td>1986 (25), P. Tarr</td>
</tr>
<tr>
<td>O157:H7 Δ10</td>
<td>EHEC</td>
<td>γ, β</td>
<td>1986 (25), 1987 (46), M. McKee</td>
</tr>
<tr>
<td>EDL933</td>
<td>EHEC</td>
<td>γ, β</td>
<td>1982 (55, 66), K. Wachsmuth</td>
</tr>
<tr>
<td>H19</td>
<td>EHEC</td>
<td>β</td>
<td>1967 (61), W. Smith</td>
</tr>
<tr>
<td>O26:H11</td>
<td>EHEC</td>
<td>β</td>
<td>1997, CDC, N. Stockbine</td>
</tr>
<tr>
<td>O111:H28</td>
<td>EHEC</td>
<td>β</td>
<td>1995, CDC, N. Stockbine</td>
</tr>
<tr>
<td>O111:NM</td>
<td>EHEC</td>
<td>α</td>
<td>1995, CDC, N. Stockbine</td>
</tr>
<tr>
<td>O157:H7</td>
<td>EHEC</td>
<td>γ</td>
<td>1997, CDC, N. Stockbine</td>
</tr>
<tr>
<td>O157:H7</td>
<td>EPEC</td>
<td>α</td>
<td>1969 (38, 63), A. Jerke</td>
</tr>
<tr>
<td>E2348/69</td>
<td>EPEC</td>
<td>α</td>
<td>1997, CDC, N. Stockbine</td>
</tr>
</tbody>
</table>

a Intimins from different organisms have been classified into several different subtypes based on antigenic and sequence divergences in the C-terminal domain of the protein (1, 45). The designations of strain E2348/69 intimin as α and strain EDL933 intimin as γ were published previously (1, 45). Intimin from strain 86-24 was cloned as O111:H28 intimin on sequence identity with the intimin from strain EDL933. We have listed the intimin from strain H19 as β because the published sequence encoding the C-terminal region of this protein is identical to the published sequences of β intimins from two other strains (3, 45). Determination of the intimin subtypes for strains 97-3256, 85-3007, and 97-3256 was accomplished by PCR analysis; in the assay, the two O111:NM strains were demonstrated to have the eae gene but the sequence did not fall into α, β, or γ subtypes (54a). Thus, the intimin subtypes of the O111:NM strains are listed here as not typable (NT).
b All EHEC strains are isolates from patients with diarrhea and/or HUS except for strain 86-24eaeΔ10, which is a derivative of strain 86-24 with an eae in-frame deletion mutation constructed in our laboratory (46), and strain EDL933, which was isolated from contaminated meat associated with an outbreak (55, 66). All strains carry the eae gene except the deletion mutant 86-24eaeΔ10 (3, 30, 46, 62a; 70). CDC, Centers for Disease Control and Prevention.

c Strain 97-3256 was determined to carry the int2 gene by PCR analysis (62a). Although most O157:H7 strains isolated thus far are EPEC, there is documentation of another O55:H7 int2 strain (67).

Plasmids encoding fragments of intimin tagged with six histidines at the N termini were constructed by standard procedures (4). For each construct, a fragment of the eae gene was cloned by PCR with plasmid pEB310 as the template. Plasmid pEB310 carries the entire eae gene from EHEC O157:H7 strain 86-24 (46). Primers included sequences for restriction endonuclease sites. The PCR fragments were digested and cloned into the BamHI and KpnI sites of the histidine fusion vectors pQE30 and pQE31 obtained from Qiagen, Inc. Inserts were confirmed by sequencing. Plasmids pMW101, pMW102, and pMW103 encode the N-terminal third (His-IntN1/3), middle third (His-IntMid1/3), and C-terminal third (His-IntC1/3) of intimin, respectively. Primers used were as follows: pMW101, GTACGGAGTTGACTGTTGTTGAGGCGCG (forward); pMW102, GTACGGATCTGTACAGGTTTCTCGGT (forward) and GTACGGTACTCTGATACAAAAATATAACCGC (reverse); pMW103, GTACGGATCTGTACAAAAACCGACCGATTAC (forward) and GTACGGTACTCTGATACAAAAACCGACCGATTAC (reverse). Plasmid pMW104 encodes the N-terminal two-thirds of intimin (His-IntN2/3). To construct plasmid pMW104, the forward primer was the same as that used to make pMW101 and the reverse primer was the same as that used to prepare pMW102. Plasmid pLG145b encodes the C-terminal half of intimin (His-IntC1/2). To construct plasmid pLG145b, the forward primer used was GTCTGAGTCACATCACTGAAAGATT CAG and the reverse primer was the same as that used to design pMW103. Plasmid pEB313, which encodes RHisEae, was described previously (47).

Affinity purification of antibodies against different fragments of intimin. Antibodies specific to different regions of the intimin protein were prepared from the goat anti-RHisEae serum. This serum contained antibodies that recognized N-terminal, central, and C-terminal portions of intimin, as observed by reactivity on an immunoblot with the various intimin protein fragments (data not shown). Affinity-purified antibodies were prepared using nickel affinity chromatography. Each intimin protein fragment coupled to Affi-Gel immunoadfinity support resin (BioRad, Hercules, Calif.) according to instructions from the manufacturer. The antigen columns were prewashed with the buffer to be used for antibody elution, 100 mM glycine (pH 2.5), to remove any contaminating proteins and then washed with the antibody elution buffer. The resin was then neutralized with Tris-HCl (pH 7.5). Passage of the sera over the immunoadfinity columns and elution of the affinity-purified antibodies were done according to protocols described by Harlow and Lane (26) and the Bio-Rad instructions. The anti-His-IntN2/3 antibody preparation was concentrated by ultrafiltration with a Microsep 30K concentrator (Pall Filtron Corp., Northborough, Mass.) to bring it to a concentration similar to that of the anti-C-terminal antibody fraction. Both antibody preparations were dialyzed against tissue culture grade PBS (BioWhittaker, Walkersville, Md.) for use in the HEp-2 cell adherence assay. The concentration of the anti-His-IntN2/3 antibody preparation was 0.27 mg/ml. The antibody preparations were also analyzed by SDS-PAGE to confirm that they had similar concentrations (data not shown). To assess blocking capacity in adherence assays (described below), equal microgram quantities of each antibody fraction were compared; PBS was added to the anti-His-IntC1/3 antibody samples so that equal volumes of the anti-N- and anti-C-terminal antibodies were mixed with bacteria. Adherence assays. Adherence of EHEC to HEp-2 (human laryngeal epithelial cell line) was assessed essentially as described previously (10, 47), with slight modifications. For the assays, 6 × 105 HEp-2 cells were seeded per well in eight-well plastic chamber slides (Naige Nunc International, Naperville, Ill.) and grown for 20 to 24 h to form subconfluent (approximately 80% confluence) monolayers. Freshly grown cortical HEp-2 cells were fixed in 2% paraformaldehyde and 0.1 M sodium phosphate buffer pH 7.2 and left overnight. The cells were then washed with PBS and used for the experiments. Colonization. Intimin has also been proposed as a vaccine candidate for EPEC (reviewed in reference 31).

Our laboratory has previously reported that sera from mice immunized with intimin could block adherence of EHEC O157:H7 strain 86-24 to HEp-2 cells compared with a preimmune serum control (47). Other studies have shown reduced adherence of EPEC to tissue culture cells in the presence of immunoglobulin fractions from human milk and colostrum; the reactivity of these fractions to an unidentified outer membrane protein of the same molecular weight as intimin suggested that anti-intimin antibodies might contribute to the blocking effect (8, 11). In the present report, we sought to (i) confirm our hypothesis that the reduction in adherence of EHEC seen in the presence of anti-intimin polyclonal serum is specifically due to anti-intimin antibodies, (ii) define the region in intimin that is important for the effects on adherence, and (iii) assess whether antisera raised against intimin from an O157:H7 strain could inhibit adherence of intimin-expressing strains of different serotypes.

MATERIALS AND METHODS

Bacterial strains and plasmids. Clinical isolates of E. coli are described in Table 1. Strain M59[prep4], obtained from Qiagen, Inc. (Chatsworth, Calif.), was used for expression and purification of the histidine-tagged intimin protein termed RHisEae (47). Because the E. coli ShD protein is known to copurify with histidine-tagged fusion proteins on metal affinity chromatography (57, 69), we used a shD::kan mutant host strain for overexpression and purification of the other His-tagged intimin protein constructs. We obtained strain RY244, which contains the shD::kan mutation, from W. D. Roof and R. Young. To construct a host strain with improved expression of the intimin fragments, we transduced the shD::kan mutation into strain UT5600 (17), and then we introduced into this strain an F- carrying a lacI' promoter gene by conjugation with strain XLI-Blue (6). The resulting strain, L172, yielded sufficient expression of all intimin fragment tested.
tubes were mixed gently three times during this period. HEp-2 cell monolayers (BioWhittaker), 1% mannose, 0.4% sodium bicarbonate) at 37°C for 35 min, and then goat anti-intimin serum, 1:2,700; goat anti-His-IntC1/3, 1:167; goat anti-His-IntN2/3, 1:167 were added. The monolayers were then washed three times with PBS. Cells were fixed and stained with the Ponceau S stain according to instructions of the manufacturer (Biochemical Sciences, Inc., Swedesboro, N.J.). To assess the effect of the purified antibodies on the adherence assay, several minor modifications were made to optimize the above procedure. First, fetal bovine serum, preheated to inactivate complement factors, was added to the adherence medium to a 0.5% final concentration. Second, the bacteria were preincubated in adherence medium for 20 to 30 min before addition of antibodies. In addition, adherent bacteria were counted as a control.

To evaluate the degree of bacterial adherence in each assay, slides were first scored for adherence by blinded microscopic observation, i.e., without a key as to which wells contained which antiserum or PBS. Following this, to quantitate the adherence of the bacteria, eight (or more) randomly selected fields from each chamber slide well that contained either preimmune serum or postimmunization serum were photographed and the number of bacteria counted. Quantitation was done by this method because, as previously reported, visual observation of the bacteria on HEp-2 cells was a more sensitive method for detection of differences between strains than viable counts of bacteria eluted from the wells (47). Since this may be due to excess bacteria that were observed to be non-specifically adherent to the plastic along the very edges of the wells, we did not include fields at the very edges of wells for quantitation. For each independent assay, the total number of HEp-2 cells counted in each set of eight photographs was counted and averaged. For each assay done with affinity-purified antibodies, photographs of 11 or 12 random fields for each well were taken; at least 160 HEp-2 cells per antibody or buffer-alone sample were counted, and at least 3,500 bacteria were counted for the anti-His-IntN2/3 samples. Statistical analysis of the data was performed as described in Tables 2 and 3.

Immunoblot analysis. Whole-cell bacterial lysates were prepared as follows. Bacteria were grown to log phase in Luria broth, harvested by centrifugation, and resuspended in 1/10 volume of medium. Sample buffer that contained SDS was added immediately, and the solution was heated for 4 min at 100°C and loaded onto polyacrylamide-SDS gels. Amounts of the lysates loaded were normalized to the optical densities at 600 nm of the log-phase cultures.

Proteins separated by SDS-PAGE were blotted onto nitrocellulose and visualized on the blot with Ponceau S stain (Sigma, St. Louis, Mo.) to ensure even loading and transfer across all lanes. The nitrocellulose membranes were blocked with 5% nonfat dried milk in PBS, washed with Tris-buffered saline (pH 7.6) and 0.25% Tween (TBST), and incubated for 2 h with primary antibody diluted in TBST. The primary antibodies were used at the following dilutions: preimmune and goat anti-intimin serum, 1:2,700; goat anti-His-IntC1/3, 1:167; goat anti-His-IntN2/3, 1:2,222. Membranes were washed in TBST and incubated for 1 h in TBST plus 10% milk and 0.1% Tween. Horseradish peroxidase-conjugated secondary antibody was used (Boehringer Mannheim). The signal was visualized by chemiluminescence with a Kodak autoradiography film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Polyclonal antisera raised against intimin from strain 86-24 reduced the adherence of this strain to HEp-2 cells. We examined the adherence of strain 86-24 to HEp-2 cells in the presence of antisera raised in a goat against histidine-tagged intimin protein RIHHisEae. The RIHHisEae protein is a histidine-tagged version of the entire intimin protein from strain 86-24 minus the N-terminal 35 amino acids, which are thought to be part of a cleaved N-terminal signal sequence (22, 47).

Antiserum to intimin and reduced adherence of EHEC 6614

<table>
<thead>
<tr>
<th>Intimin fragment</th>
<th>Sample antibody (animal source)</th>
<th>Reduced adherence in the presence of the antibody samplea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intimin1–5157</td>
<td>Polyclonal serum (goat)</td>
<td>+</td>
</tr>
<tr>
<td>His-IntC1/2</td>
<td>Polyclonal serum (rabbit)</td>
<td>+</td>
</tr>
<tr>
<td>His-IntC1/3</td>
<td>Polyclonal serum (mice)</td>
<td>−b</td>
</tr>
<tr>
<td>His-IntN2/3</td>
<td>Purified antibody fraction (goat)</td>
<td>−</td>
</tr>
<tr>
<td>His-IntC1/3</td>
<td>Purified antibody fraction (goat)</td>
<td>+a</td>
</tr>
</tbody>
</table>

a, +, reduced adherence observed; −, no inhibition of adherence observed.

The average reduction in adherence in the presence of anti-intimin1–5157 polyclonal serum relative to preimmune serum (from three assays) was 8.1-fold; bacterial counts are presented in Table 3 in the context of other experiments. For the other polyclonal sera, bacterial counts were not determined; relative levels of adherence compared to preimmune sera were assessed microscopically.

Serum from three of the four immunized mice reduced adherence of strain 86-24. The level of adherence in the presence of antibodies to the C-terminal third of intimin (anti-His-IntC1/3) was an average of 4.4-fold lower than that in the presence of antibodies to the N-terminal region (anti-His-IntN2/3). To determine the average fold reduction, three independent assays were performed; for each assay, bacteria and HEp-2 cells were counted in 11 or 12 microscope fields per sample. Each field contained at least 10 HEp-2 cells, although not every HEp-2 cell contained adherent bacteria. Thus, the following data for the three assays are presented as the average of the average numbers of bacteria (± two times the standard errors of the means) per 10 HEp-2 cells (anti-His-IntN2/3-anti-His-IntC1/3): 230 ± 24/101 ± 21, 222 ± 36/43 ± 13; 253 ± 30/45 ± 9. In each experiment, the difference between adherence levels in the presence of the two antibody fractions was significant (Student’s unpaired t test; P < 0.001).

Polyclonal sera raised against C-terminal fragments of intimin reduced adherence of strain 86-24. As one way to investigate the importance of the C-terminal region of intimin in the adherence-reducing capacity of anti-intimin antisera, we immunized a rabbit with theHis-tagged C-terminal half fragment (His-IntC1/2) and four mice with the tagged C-terminal one-fifth fragment (His-IntC1/5). Intimin fragments are shown in Fig. 1. As assessed by microscopic observation, serum samples from the rabbit immunized with His-IntC1/2 reduced adherence of 86-24 to a greater extent than did the preimmune serum (Table 2). The antisera from three of the four immunized mice also reduced adherence of 86-24 to a greater extent than did the preimmune sera from the respective mice, although to different degrees (Table 2). No difference between pre- and postimmunization sera from one of the mice was observed; this result is addressed in Discussion.

An affinity-purified antibody fraction containing antibodies to the C-terminal third of intimin reduced adherence of strain 86-24 to HEp-2 cells. Since serum contains other factors in addition to antibodies, we sought to test our hypothesis that the reduction in adherence in the presence of anti-intimin1–5157 antisera was due to the presence of anti-intimin antibodies. Additionally, we sought to investigate by another method the importance of antibodies to the C-terminal region of intimin, suggested by our results above, for the effect on adherence. We thus purified two antibody fractions from the goat anti-intimin1–5157 antisera for use in the adherence assays: an anti-
The anti-His-IntC1/3 antibody fraction did not show a consistent effect on adherence, the 2.4- and 4-μg amounts both showed reproducible effects, with the effect of 4 μg about twice that of 2.4 μg. Data for 4 μg of the anti-His-IntC1/3 antibody fraction are presented in Table 2; in three independent experiments, performed on different days, the anti-His-IntC1/3 antibody fraction reduced adherence of strain 86-24 to HEp-2 cells by an average of 4.4-fold compared to adherence levels seen in the presence of the anti-His-IntN2/3 antibodies. The results observed with the fraction of antibodies containing reactivity to the C-terminal third of intimin indicate that anti-intimin antibodies can reduce adherence to HEp-2 cells and that epitopes in the C-terminal third of intimin are critical for this effect.

Immunoblot analysis with whole-cell lysates of *E. coli* strains demonstrated the presence of reactivity in these antibody fractions to the 97-kDa intimin protein in the pathogenic *E. coli* examined. The immunoblots revealed additional reactivity to one other band, of approximately 15 kDa, that was present in both clinical and nonpathogenic laboratory strains. However, for reasons discussed below, we think that the antibodies to the C-terminal domain of intimin caused the reduction in adherence of strain 86-24.

Polyclonal antisera raised against intimin from O157:H7 strain 86-24 reduced the HEp-2 cell adherence of another O157:H7 strain and that of some, but not all, strains of other serotypes. Next, we sought to determine whether antisera raised against intimin from an O157:H7 strain could reduce adherence of EHEC strains of other serotypes. For these experiments, we chose to analyze (i) an additional strain of the O157:H7 serotype to complement our studies on 86-24; (ii) an O55:H7 strain, since this serotype is closely related to O157:H7 strains (54, 67, 68); and (iii) strains from the O26 and O111 serogroups, since these are the most frequent non-O157:H7 isolates from hemorrhagic colitis and HUS patients (58). We analyzed two independently isolated O26:H11 strains and two independently isolated O111:NM strains. Additionally, we tested one EPEC strain, O127:H6 strain E2348/69. All of the strains were isolated either from human patients or from contaminated food associated with an outbreak and are described in Table 1. Since some of these strains, especially the recent isolates, had not been characterized with respect to A/E lesion formation, we first tested all of the strains in HEp-2 cell assays for the capacity to rearrange cellular actin, a hallmark of the A/E lesion and thus of intimin function. We used the standard fluorescence actin staining (FAS) test (33); as expected, all of these strains were FAS positive (data not shown).

Trial assays were done to assess the adherence capacity of each *eae*⁺ strain. Most of the strains displayed a greater level of adherence to HEp-2 cells than did strain 86-24 (data not shown). For more convenient comparison with strain 86-24, cultures of these other strains were diluted prior to the assays, as noted in Table 3. Additionally, we further titrated down the number of bacteria in an attempt to optimize the adherence-blocking effects for strains that did not show a strong effect initially. To observe a reduction of adherence by the goat anti-intiminO157:H7 serum, some strains also required the addition of more serum than was necessary to see an effect on strain 86-24. Data are presented in Table 3 for three independent assays performed on different days.

Addition of anti-intiminO157:H7 antisera to the HEp-2 cell assay mixture reduced adherence of some of the strains compared with their adherence in the presence of preimmune serum; despite some day-to-day variation in the assay, the greatest fold reductions were seen with the two O157:H7 strains and the EHEC O55:H7 strain (Table 3). Somewhat lesser effects, on average, were seen for strain 85-3007 (O111:NM) and for
TABLE 3. Adherence of strains of different serotypes in the presence of polyclonal serum that contains antibodies against intimin_{O157}

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Assay</th>
<th>Preimmune serum</th>
<th>Anti-intimin serum (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>86-24</td>
<td>O157:H7</td>
<td>I</td>
<td>106 ± 25</td>
<td>10 ± 5 (8.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>50 ± 13</td>
<td>6 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>158 ± 26</td>
<td>34 ± 10</td>
</tr>
<tr>
<td>EDL933</td>
<td>O157:H7</td>
<td>I</td>
<td>263 ± 66</td>
<td>30 ± 10 (5.7)</td>
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<tr>
<td></td>
<td></td>
<td>II</td>
<td>195 ± 25</td>
<td>43 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>220 ± 39</td>
<td>60 ± 21</td>
</tr>
<tr>
<td>97-3256</td>
<td>O55:H7</td>
<td>I</td>
<td>109 ± 22</td>
<td>11 ± 6 (10.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>187 ± 49</td>
<td>11 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>136 ± 34</td>
<td>28 ± 10</td>
</tr>
<tr>
<td>85-3007</td>
<td>O111:NM</td>
<td>I</td>
<td>57 ± 19</td>
<td>16 ± 12 (3.0)</td>
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<tr>
<td></td>
<td></td>
<td>II</td>
<td>29 ± 9</td>
<td>17 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>61 ± 19</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>H19</td>
<td>O26:H11</td>
<td>I</td>
<td>58 ± 13</td>
<td>25 ± 7 (1.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>67 ± 17</td>
<td>37 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>65 ± 15</td>
<td>41 ± 16</td>
</tr>
</tbody>
</table>

a The numbers of bacteria added to the assay mixtures with respect to strain 86-24 (n = 1.2 × 10^8) were as follows: EDL933, n/2; 97-3256, n/15; 85-3007, n/6; H19, n/5. Serum dilutions were 1:140 for strains 86-24 and EDL933 and 1:90 for strains 97-3256, 85-3007, and H19.

b Three independent assays were performed. For each assay, the bacteria and HEp-2 cells were loaded in 8 to 14 microscope fields per sample. The total number of HEp-2 cells counted in the set of fields observed for each sample was at least 100. The difference between the levels of adherence of the O157:H7 and O55:H7 strains in the presence of preimmune serum and polyclonal anti-intimin serum was significant, as calculated by Student’s unpaired t test; P < 0.001 for each assay. For O111:NM strain 85-3007, the difference between the sera in assay II was not significant (P > 0.05); the P values for assays I and III were <0.01 and <0.001, respectively. For O26:H11 strain H19, the P values were <0.001, <0.02, and <0.05, respectively, for assays I to III.

c The fold reduction in adherence in the presence of anti-intimin antiserum was calculated with respect to the preimmune serum control and was computed before rounding the numbers for each sample. The average fold reductions and ranges for the three assays are presented. For strains 95-3208, 97-3250, and E2348/69 there was no anti-intimin_{O157}-specific reduction in adherence.

strain H19 (O26:H11). The reduction in adherence seen with these strains was readily apparent by microscopic observation even before bacterial counts were assessed. However, there was no specific anti-intimin_{O157} serum effect on adherence of strains 95-3208 (EHPEC O111:NM), 97-3250 (EHPEC O26: H11), and E2348/69 (EPEC O127:H6), as assessed by microscopic observation, in at least two independent assays in which different amounts of sera and bacteria were tested. Thus, adherent bacteria were not counted for these strains.

We also tested our rabbit anti-His-IntC1/2 polyclonal antisera for the capacity to reduce adherence of the strains for which an effect was seen with the anti-intimin_{O157} serum, as described above. These included O157:H7 strains 86-24 and EDL933, O55:H7 strain 97-3256, O111:NM strain 85-3007, and O26:H11 strain H19. In support of the above results, the rabbit polyclonal serum reduced adherence of these strains compared to the preimmune serum control, as assessed by microscopic observation; the effects on adherence were most readily observed with the two O157:H7 strains, 86-24 and EDL933 (data not shown).

Immunoblot analysis with the affinity-purified antibody preparations demonstrated the absence of the 30- and 18-kDa bands that were observed with the crude serum; thus, reactivity to these proteins did not contribute to the reduction in adherence of strain 86-24 in the presence of the purified anti-His-IntC1/3 preparation. As noted above, there was reactivity to the 15-kDa band in our purified antibody preparations (data not shown). The His-IntC1/3 antibody preparation contained somewhat stronger reactivity to this band than the His-IntN2/3 antibody preparation. Since this protein was present in all strains examined, including the nonadherent eae mutant derivative of 86-24 and nonpathogenic laboratory E. coli strains (Fig. 2B), it does not function as an adherence factor in the HEp-2 cell assay. Unlike the significant antigenic divergence observed in the C-terminal third of intimin among different strains, the intensities of the 15-kDa bands in all strains, including nonpathogenic laboratory E. coli were similar, as assessed by immunoblotting with polyclonal serum and with the affinity-purified antibody fractions (Fig. 2B and data not shown). Thus,
FIG. 2. Immunoblot analysis of intimin from whole-cell lysates of different E. coli strains with antibodies raised in a goat against intimin from O157:H7 strain 86-24. The strains analyzed were as follows (lanes 1 to 8 are EHEC strains): lane 1, 86-24 (O157:H7); lane 2, 86-24 ΔeaeO157; lane 3, EDL933 (O157:H7); lane 4, 97-3256 (O55:H7); lane 5, 85-3007 (O111:NM); lane 6, H19 (O26:H11); lane 7, 86-24. The strains analyzed were as follows (lanes 1 to 8 are EHEC strains): lane 1, 86-24 (O157:H7); lane 2, 86-24 ΔeaeO157; lane 3, EDL933 (O157:H7); lane 4, 97-3256 (O55:H7); lane 5, 85-3007 (O111:NM); lane 6, H19 (O26:H11); lane 7, 97-3250 (O26:H11); lane 8, 95-3208 (O111:NM); lane 9, EPEC strain E2348/69 (O127:H6); lane 10, nonpathogenic E. coli K12 strain UT5600. (A) Reactivity of preimmune serum. (B) Reactivity of polyclonal serum raised against intiminO157. For blots A and B, whole-cell lysates were run on 15% polyacrylamide–SDS gels for this figure to display all of the reactive bands. Identical serum dilutions were used for the preimmune and anti-intimin antisera, and blots A and B were exposed to film simultaneously for chemiluminescent detection. (C) Reactivity of purified antibodies against the N-terminal two-thirds (top) and C-terminal third (bottom) of intimin. Bacterial lysates were run on 10% polyacrylamide–SDS gels for the blots shown here. Analysis of higher-percentage gels showed additional reactivity to a 15-kDa band in all lanes (data not shown). All immunoblots were scanned with a Hewlett-Packard ScanJet 4C scanner, and the images of the four blots were arranged for the figure and labeled with Adobe Photoshop version 5.0.2 for Macintosh.

In this study, we examined the adherence to HEp-2 cells of O157:H7 strain 86-24 and pathogenic eaeE. coli of other serotypes in the presence of antisera that contained antibodies to intiminO157. We observed a reduction in adherence of O157:H7 strain 86-24 in the presence of crude polyclonal anti-intimin antiserum and in the presence of a purified antibody fraction that contained antibodies to the C-terminal third of intimin. The adherence of some, but not all, of the other EHEC strains tested was also reduced in the presence of the polyclonal anti-intiminO157 antiserum compared with that in the presence of the preimmune serum. Immunoblot analysis revealed antigenic divergence in the C-terminal domains of the intimins from these strains. Although the serum recognized several other E. coli proteins in addition to intimin, these other proteins were not specific to pathogenic E. coli. Furthermore, the fold reduction in adherence in the presence of serum containing anti-intiminO157 antibodies was greatest for strains that expressed an intimin protein that was very similar or identical in the C-terminal domain to intimin from strain 86-24. Conversely, by immunoblot analysis, the bands detected for each of the O26, O111, and O127:H6 strains with antibodies to the C terminus were much less intense than the bands detected with the antibodies to the N terminus. This pattern of differential immunoreactivity between the two domains indicated more divergence in the C terminus and correlated with lesser effects (or no effect) of the anti-intiminO157 sera on adherence of these strains. The C-terminal cross-reactivity detected in these strains by immunoblotting may represent some epitopes in the C terminus that are not critical for adherence or that are not accessible to antibody binding when the protein is in the bacterial outer membrane.

There are data from several laboratories showing the importance of the C-terminal third of intimin for the interaction of EPEC and EHEC with host epithelial cells (13, 18, 19, 27, 32, 40). Thus, the decreased EHEC adherence that we observed in the presence of antibodies to the C-terminal third of intimin may be due to the blocking of functional regions of this domain of intimin, preventing interaction either between intimin and the translocated intimin receptor protein Tir or between intimin and a host cell protein. It is of interest that host cell invasion by a different pathogen, the Yersinia species, can be blocked by antibodies to the Yersinia protein invasin, which shows some homology to the intimin proteins (28, 30, 35, 36, 70). While we did not observe decreased adherence in the presence of antibodies to the N-terminal two-thirds of intimin, our data do not rule out the possibility that a high titer of antibodies specific to a particular epitope within this region of the protein might functionally or stereically reduce adherence.

The relative effects of our anti-intiminO157 serum on adherence of strains of different serotypes correlate with published sequence divergence in the C-terminal domain of intimin and with evolutionary relationships among strains of these serotypes. The trends we observed showed the greater fold reductions of adherence of the two O157:H7 strains and the O55:H7 strain and lesser effects with the other strains. Sequence analysis showed 100% amino acid identity in the regions comprising the C-terminal thirds of the 86-24 and EDL933 intimin proteins (21, 45, 46, 70). Although the eae gene from the EHEC O55:H7 strain examined in this study has not yet been

the adherence-reducing capacity of the polyclonal antiserum for the strains of different serotypes correlated only with the pattern of antigenic cross-reactivity in the C-terminal domain of intimin.

DISCUSSION

In this study, we examined the adherence to HEp-2 cells of O157:H7 strain 86-24 and pathogenic eaeE. coli of other serotypes in the presence of antisera that contained antibodies to intiminO157. We observed a reduction in adherence of O157:H7 strain 86-24 in the presence of crude polyclonal anti-intimin antiserum and in the presence of a purified antibody fraction that contained antibodies to the C-terminal third of intimin. The adherence of some, but not all, of the other EHEC strains tested was also reduced in the presence of the polyclonal anti-intiminO157 antiserum compared with that in the presence of the preimmune serum. Immunoblot analysis revealed antigenic divergence in the C-terminal domains of the intimins from these strains. Although the serum recognized several other E. coli proteins in addition to intimin, these other proteins were not specific to pathogenic E. coli. Furthermore, the fold reduction in adherence in the presence of serum containing anti-intiminO157 antibodies was greatest for strains that expressed an intimin protein that was very similar or identical in the C-terminal domain to intimin from strain 86-24. Conversely, by immunoblot analysis, the bands detected for each of the O26, O111, and O127:H6 strains with antibodies to the C terminus were much less intense than the bands detected with the antibodies to the N terminus. This pattern of differential immunoreactivity between the two domains indicated more divergence in the C terminus and correlated with lesser effects (or no effect) of the anti-intiminO157 sera on adherence of these strains. The C-terminal cross-reactivity detected in these strains by immunoblotting may represent some epitopes in the C terminus that are not critical for adherence or that are not accessible to antibody binding when the protein is in the bacterial outer membrane.

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cloned, the eae genes from two other O55:H7 strains have been sequenced (42, 45) and show approximately 99% identity in the C-terminal region to the intimin from strain 86-24. Among the other strains we tested, sequence information is available for the eae genes of O26:H11 strain H19 and EPEC O127:H6 strain E2348/69 (3, 30). These sequences show 59 and 55% amino acid identity, respectively, to the 86-24 sequence in the C-terminal thirds of the proteins. Additionally, consistent with our results, recent studies have identified different subtypes of intimin C-terminal domains based on antigenic variation and PCR analysis (1, 45). As noted in Table 1, the intimins from the O157:H7 and O55:H7 strains used in the present study were of the γ subtype; the intimins from the other strains fell into different subtypes.

Our laboratory and others have proposed intimin as a candidate for an anticolonization vaccine strategy for EHEC (7, 12, 31). Such a vaccine could theoretically be used in humans or in cattle, since cattle are the primary reservoir for STEC that infects humans. Recently, a strategy employing antibodies to colonization factor antigens to protect against oral challenge with another pathogen, enterotoxigenic E. coli, was shown to be successful in a volunteer study (20). A different approach toward a vaccine for EHEC currently under investigation by other researchers is the use of O157:H7 lipopolysaccharide as a candidate antigen to elicit antibodies that may lyse the bacteria in the presence of complement or sterically block adhesion (34, 52).

Circumstantial evidence that is consistent with the hypothesis that an immune response to intimin in humans might confer protection against infection with eae+ E. coli exists. As noted above, intimin is immunogenic in humans (29, 39, 43, 44, 47, 50, 65). Additionally, in one study of volunteers experimentally infected with EPEC, 1 of the 10 volunteers did not develop diarrhea; this was the only individual who had prechallenge serum antibodies to a 94-kDa protein later shown to be intimin (39, 50). Another volunteer study that involved challenge with EPEC and subsequent rechallenge did not observe a correlation between antibody titer to intimin and severity of disease upon rechallenge (15). In that model of infection, however, the antibody response to intimin may not have been sufficient. We note in this context that one of the four mice immunized for this study with the fragment comprising the C-terminal third of intimin did not show an adherence-blocking response. Thus, optimization of the immunization protocol might be necessary to elicit a more uniform immune response. We additionally immunized one animal with RHisEae protein that had been excised from an SDS-PAGE gel, and we found that the serum obtained did not inhibit adherence of 86-24 (data not shown). This result may hint that conformational epitopes, disrupted in this case by SDS, play a role in eliciting a blocking response. Since the anti-His-IntC13 antibody-containing fraction from the polyclonal serum was purified on an immunoaffinity column that had been prewashed with acidic buffer, we infer that at least some relevant epitopes of intimin may be stable to exposure to acidic conditions, a property that would be useful in an oral vaccine.

Our data suggest that serum raised against intiminα157 may reduce colonization of O157:H7 and closely related strains but that a higher titer of anti-intimin antibodies may be required to block colonization of strains that display greater adherence capacity due to as yet unidentified regulatory or structural factors. We observed a general correlation between the antigenic similarity in the C-terminal domain of intimin and the capacity of antiserum containing antibodies to intiminα157 to reduce adherence of heterologous strains. Thus, sequence divergence at the C-terminal domain may preclude development of a broadly cross-protective vaccine with intiminα157 as the antigen. While O157:H7 is the most frequent cause of STEC-mediated disease in many countries, one way to encompass more of the non-O157 eae-producing strains in a single vaccine might be to consider a multivalent vaccine with intimin fragments from several major clinically relevant serotypes.

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