HOST RESPONSE TO INFECTION

Antibody response of patients infected with verocytotoxin-producing *Escherichia coli* to protein antigens encoded on the *LEE* locus


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Sera from patients infected with verocytotoxin-producing *Escherichia coli* (VTEC) O157, from patients with antibodies to *E. coli* O157 lipopolysaccharide (LPS) and from healthy controls were examined for antibodies to proteins involved in expressing the attaching and effacing phenotype. After SDS-PAGE, purified recombinant intimin, EspA-filament structural protein, translocated protein EspB and three separate domains of the translocated intimin receptor (Tir) were tested for reaction with patients' sera by immunoblotting. An ELISA was also used to detect antibodies to intimin in sera from *E. coli* O157 LPS antibody-positive individuals. Seven of nine culture-positive patients and one control patient had antibodies to EspA. Five of these patients and two controls had serum antibodies to the intimin-binding region of Tir, whereas none of the sera contained antibodies binding to either of the intracellular domains of Tir. By immunoblotting, 10 of 14 culture-positive patients had antibodies to the conserved region of intimin, eight of whom were infected with *E. coli* O157 phage type 2. Thirty-six of 60 sera from culture-negative but *E. coli* O157 LPS antibody-positive patients had antibodies to intimin as determined by ELISA. The secreted proteins are expressed in vivo during infection and are considered as pathogenic markers. Antibodies to these proteins may form the basis of a serodiagnostic test for the detection of patients infected with VTEC which carry the locus for the enterocyte effacement pathogenicity island and provide an adjunct test to the established serological tests based on VTEC LPS.

Introduction

Strains of *Escherichia coli* that produce verocytotoxin (VT) and belong to serogroup O157 are emerging zoonotic pathogens of worldwide importance and a major cause of haemolytic uraemic syndrome (HUS) [1, 2]. Studies of the pathogenesis of infections caused by VT-producing *E. coli* (VTEC) O157 have demonstrated that the ability of strains to produce VT during infection, as demonstrated by its presence in faeces [3, 4], is a key pathogenicity factor.

An integral part of the pathogenesis of infection with VTEC is their ability to adhere to gut mucosa. In common with certain strains of enteropathogenic *E. coli* (EPEC), VTEC O157 adhere to the human gut and cause attaching and effacing (A/E) lesions [5]. The first gene to be associated with A/E activity was the eae gene encoding the intimate bacterial adhesin, intimin [6]. The cell-binding activity of the intimin family of proteins is localised to the C-terminal 280 amino acids (Int280) [7, 8], and four different Int280 types, designated α, β, γ and δ, have been described [9].

The eae gene was shown subsequently to be part of a large (c. 35 kb) pathogenicity island, the locus for enterocyte effacement (*LEE*) [5]. In addition to intimin, the *LEE* encodes a type III secretion system, an intimin receptor, Tir/EspE, and three EPEC secreted proteins (EspS), EspA, EspB and EspD, required for signal transduction in host cells and A/E lesion formation [5]. One unexpected recent finding was that EspA is a structural protein and a major component of a large filamentous organelle that is present transiently on the
bacterial surface and which interacts with the host cell during the early stage of A/E lesion formation [9]. EspA filaments may contribute to bacterial adhesion because they form interactions with host cells. More importantly, however, they appear to be a component of a translocation apparatus and are essential for the translocation of EspB and also Tir to host cells [9]. Recently, gel overlays and the yeast two hybrid system have been used to localise the intimin-binding region of Tir to a stretch of 107 amino acids between the two trans-membrane domains [9].

Patients infected with VTEC O157 are known to produce serum and salivary antibodies to LPS [10, 11], and this has formed the basis for the serodiagnosis of the infection [10]. Furthermore, patients infected with E. coli O157 and other VTEC may produce antibodies to one or more of the secreted proteins [12].

In a recent study, Paton et al. [13] showed that patients infected with E. coli O157 produced antibodies to Tir expressed by strains of E. coli belonging to serogroups O111 and O157. In the present study, sera from patients who were culture-positive for VTEC O157 or had serum antibodies to the O157 LPS, or both, were used to determine the antibody response to purified preparations of recombinant intimin, EspA, EspB, and three different regions of Tir.

Materials and methods

Preparation of recombinant intimin, EspA, EspB and Tir

A fragment encoding the 280 amino acids residues Gly 387–Lys 666, upstream of the cell-binding domain of eae from EPEC strain E2348/69 (O127:H6) was amplified by PCR, with primers Int-Fc: 5′- CGG GAT TAC GAT CGT CAT 3′ and Int- Rc: 5′- TGC ACA TC 3′, amplified by PCR, with primers Int-Fc: 5′- CGG GAT TAC GAT CGT CAT 3′ and Int- Rc: 5′- TGC ACA TC 3′, by 30 amplification cycles of 95°C for 20 s, 50°C for 45 s and 74°C for 1 min. This sequence was then subcloned into the EcoRI and HindIII site of pET28a (Novagen Biotechnology) and the recombinant plasmids were transformed into E. coli BL21. The pET28a vector directs expression of cloned genes from an inducible T7 promoter as His-tag fusions. Induced cultures were sonicated and the supernatant was collected and purified on nickel affinity columns. DNA segments encoding EspA (PCR primers F-5′- CGG AGT ACT TCG ACA TC 3′ and R-5′- TTA TTT ACC AAG GGA TAT 3′), EspB (PCR primers F-5′- CTG AAT TCA CGC TGG TCA TTT ATC AGC CTT AAT CTC TTC GGG ATC GAT TAC CGT CAT 3′ and R-5′- CGG GAT CCT TAA TGC ACT GGT TT 3′), Tir-N (N-terminal region; PCR primers F-5′- CGG GAT CCA TGC CTA TGG GTA ACC TCG TGG G 3′ and R-5′- CGG GAT CCT TAA TTT GGT GAA GTA TG 3′) and Tir-M (middle region between the two putative trans-membrane domains; PCR primers F-5′- GGA ATT CCA GGC GTT GGC TTT GAC A 3′ and R-5′- CGG GAT CCT TAC GAT GAA AGC TGT AAT TC 3′) and Tir-C (C-terminal region; PCR primers F-5′- CGG GAT CCC CCG CAG CAG A AC AGA CAA C 3′ and R-5′- CGG GAT CCT TAA ACG AAA CGT ACT GGT CC 3′) were amplified in a similar way with EPEC E2348/69 DNA as template. These fragments were also cloned into pET28a in E. coli BL21 and the His-tagged polypeptides were purified.

Sera

Fourteen sera were from patients with faecal carriage of VTEC O157 expressing VT2 only, who had serum antibodies to the O157 LPS antigens. Sixty sera were from patients from whose faeces VTEC O157 was not isolated, but who had serum antibodies to E. coli O157 LPS. Of these 60 patients, 27 were male (mean age 4.9 years) and 33 were female (mean age 3.4 years) and 31 of them (11 males and 20 females) were diagnosed clinically as having HUS. Twenty sera, obtained from apparently healthy individuals, were obtained from the blood transfusion service, London and were used as controls.

ELISA

The wells of microtitration plates were coated with 0.2 μg of intimin in 100 μl of coating buffer (1.59 g Na2CO3 and 2.93 g NaHCO3/L of distilled water, pH 9.6) and sera diluted 1000-fold in phosphate-buffered saline were added. Antigen–antibody complexes were detected by alkaline phosphatase-conjugated goat anti-human polyclonal antiserum (Sigma; Product code A5034). The enzyme substrate, p-nitrophenol phosphate (Sigma) 1 mg/ml in diethanolamine buffer (diethanolamine 97 ml, MglCl6H2O 100 mg/L of distilled water, pH 9.8) was added and, after incubation for 30 min at room temperature in the dark, the resultant yellow colour was quantified by measuring its absorbance at 405 nm.

SDS-PAGE and immunoblotting

SDS-PAGE was performed by the method of Laemmli [14] with an Atto mini-gel system (Genetic Research Instrumentation). Preparations containing 10 μg of protein in 5 μl of SDS-PAGE solubilisation buffer – glycerol 100 g, β-mercaptoethanol 50 ml, bromophenol blue 0.1 g/L in 62.5 mM Tris (hydroxymethyl) methylamino-HCl, pH 6.8 – were incubated at 100°C for 5 min before loading on to gels comprising an acrylamide 4.5% stacking gel and an acrylamide 12.5% separation gel. After electrophoresis at 50 mA for 30 min, gels were either stained with Coomassie Brilliant Blue or the proteins were transferred to nitrocellulose sheets by electrophoresis at 0.5A for 1.5 h by the method of Towbin et al. [15]. After transfer, immunoblots were cut into strips and each profile was treated with patients’ sera (10 μl). Anti-
body–antigen complexes were detected with a goat anti-human polyvalent antibody conjugated with alkaline phosphatase (Sigma) and an enzyme substrate buffer comprising nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate [12].

Results

Previous reports have shown differential reactivity of human and animal sera with intimin from different VTEC and EPEC isolates [9, 13, 14]. To obtain a recombinant intimin antigen reactive with broad-spectrum VTEC-infected human sera, the nucleotide sequence for the 280 amino acids, residues Gly 387–Lys 666, upstream of the cell-binding domain of eae from EPEC strain E2348/69 (encoding intimin α), was amplified and cloned in pET28a, producing Int280387–666. The amino-acid sequence of this region from EPEC O127 and VTEC O157 [9] is highly conserved, sharing 88% identity and 90% similarity. The Int280387–666 polypeptide was over-expressed in E. coli BL21 and the protein was purified. On SDS-PAGE, the Int280387–666 domain migrated as a band of 38 kDa (Fig. 1, lane 1). By immunoblotting, it was found that sera from 10 of 14 patients with E. coli O157 in their faeces but none of the 20 sera from the healthy controls contained antibodies to this intimin preparation (for an example see Fig. 2, lane 1; Table 1).

Sera from 60 culture-negative patients with antibodies to the LPS of E. coli O157 and 20 sera from healthy controls were examined by ELISA for antibodies to intimin. Control sera gave a mean ELISA OD405 value of 0.31 (σ = 0.1). In all, 36 patients' sera of which 20 (56%) were from patients diagnosed with HUS, were shown to have ELISA OD405 values >0.41, the mean control value plus 1 SD. The recombinant protein EspA, a filamentous structural protein associated with a filamentous structure involved in bacterial cell adhesion and protein translocation [9], migrated with an apparent molecular mass of 28 kDa (Fig. 1, lane 2). Immunoblotting sera against this antigen revealed EspA antibodies in seven of the nine patients infected with E. coli O157 and in one of 20

Table 1. Serum antibody response of patients with faecal VTEC O157 to intimin, EspA, EspB, Tir-N, Tir-C and Tir-M

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>PT</th>
<th>VT</th>
<th>Intimin</th>
<th>EspA</th>
<th>EspB</th>
<th>Tir-N</th>
<th>Tir-C</th>
<th>Tir-M</th>
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<tr>
<td>A*</td>
<td>F</td>
<td>4</td>
<td>2</td>
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<td>+</td>
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<td>B*</td>
<td>M</td>
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<td>F</td>
<td>2</td>
<td>21/28</td>
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<td>–</td>
<td>NA</td>
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<td>NA</td>
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<tr>
<th>PT, phage type; VT, VT type; NA, not available; +, antibody present.</th>
<th>Serum antibodies to</th>
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<td>A* Patients with HUS.</td>
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Fig. 1. SDS-PAGE gel showing recombinant preparations of intimin (arrowed, lane 1), EspA (2), EspB (3), Tir-M (4), Tir-N (arrowed, 5), Tir-C (6). Mol. wt markers (kDa) are shown on the left.
control sera (Fig. 2, lane 2). In contrast to EspA, the recombinant protein EspB is an effector protein translocated into the host cell cytoplasm and membrane (Fig. 1, lane 3). Immunoblotting the sera against EspB demonstrated antibodies in two of the seven patients infected with E. coli O157 and in three of 20 control sera (for example see Fig. 2, lane 3; Table 1).

To localise the intimin-binding region(s) of Tir, three fragments of Tir, corresponding to the amino-terminal region (Tir-N), the carboxyl-terminal region (Tir-C) and the middle region localised between the two putative trans-membrane domains (Tir-M), were subcloned and used in intimin-binding assays. The intimin-binding region of Tir was Tir-M, suggesting that this region is exposed at the host cell membrane while the amino- and carboxyl-terminal domains are intracellular. Indeed, tests with polyclonal antisera confirmed that both Tir-N and Tir-C are intracellular [9]. The Tir domains M, N and C were shown to be of masses 24, 28 and 30 kDa, respectively (Fig. 1, lanes 4–6), and five of eight patients infected with E. coli O157 and two of 20 control sera were shown by immunoblotting to contain antibodies to Tir-M (Fig. 2, lane 4). Serum antibodies were not detected to preparations of Tir-N and Tir-C in any of the patient or control sera (for examples see Fig. 2, lanes 5 and 6; Table 1).

Discussion

In this study, recombinant preparations of the proteins intimin, EspA, EspB and Tir, prepared from EPEC strain E2348/69 (O127), were examined for reaction with sera from patients infected with VTEC O157. Although advances have been made in studying the immune response to VTEC by developing assays to detect antibodies to LPS [10, 11] and secreted LEE-encoded VTEC proteins [12] in sera from patients with HUS, this study is believed to be the first to use recombinant purified virulence factors as antigens to determine antibody responses. Preparations of intimin and Tir N migrated in SDS-PAGE gels as more than one band. This has been reported previously by others [8]. The other bands are thought to be protein degradation products.

Of the 14 sera from patients who were culture-positive for VTEC O157 and had antibodies to O157 LPS, 10 contained antibodies reactive with intimin by blotting. These were from patients of whom eight were infected with VTEC O157 phage type 2. Of the four patients whose sera had no intimin antibodies, three were infected with VTEC O157 belonging to phage type 28 and the fourth was phage type 2. Although intriguing, the significance of this observation is at present unknown. Thirty-six of the 60 sera from patients with antibodies to E. coli O157 LPS were also shown to contain antibodies to intimin. As these sera were obtained from culture-negative patients, the phage types of the infecting bacteria were not known. However, as with sera from culture-positive patients, not all patients produced antibodies to this protein.

Because only small amounts of serum were available, reactivity with the other virulence factors could not be determined for all the sera. However, seven of nine sera were found to contain EspA antibodies. Importantly, all the EspA-positive sera also contained intimin antibodies. Both intimin and EspA are exposed on the bacterial cell surface [16] as an outer-membrane protein and filamentous structure, respectively [9]. It should be noted that none of the 20 control sera contained antibodies to intimin and only one had antibodies directed against EspA. In contrast, EspB antibodies were found in only one of the patients’ sera and in three of the 20 controls. However, this may reflect the low level of sequence similarity between EPEC and VTEC EspB.

Recently, the intimin-binding region of Tir was localised, by gel overlays and the yeast two hybrid system, to a stretch of 107 amino acids between the two putative trans-membrane domains of the polypeptide (designated Tir-M). Immunofluorescence staining with polyclonal antisera to the amino- and carboxyl-termini (Tir-N and Tir-C, respectively) demonstrated that these two regions are located within the infected host cell where they can induce focusing of the polymerised actin and other cytoskeletal proteins to produce the characteristic pedestal-like structure. In this study, serum antibodies reactive with Tir-N or -C were not found. In contrast, five of the nine patients’ sera and only two of the 20 controls contained antibodies to Tir-M, which is predicted to be surface accessible, following translocation, on the mammalian cells. However, it is important to
note that the Tir fragments used in this study were made from the EPEC prototype strain E2348/69 and, although both Tir-N and Tir-M share a high level of sequence similarity in EPEC and VTEC (75% and 77%, respectively), Tir-C shares only 52% sequence similarity. Although it has been shown previously that Tir-C, like Tir-N, is intracellular following translocation, it cannot be excluded that lack of antibodies against Tir-C is a consequence of a low level of cross-reactivity between EPEC and enterohaemorrhagic E. coli. All the human sera that contained Tir antibodies also reacted with intimin and of the eight patient sera tested against intimin, EspA and Tir-M, five had antibodies to all three.

The isolation and characterisation of VTEC from patients' stools is usually performed to identify the cause of disease, while the detection of vtx genes or free VT in faeces can indicate the possible involvement of VTEC [4]. Because it is not always possible to obtain evidence of VTEC infection by these methods, serological tests based on purified LPS have been developed to provide evidence of infection with E. coli O157 and other serogroups [10]. The present study provides evidence that EspA, Tir and intimin are expressed during infection with VTEC and that these proteins in general, and intimin in particular, may be useful for the serodiagnosis of VTEC infection. Immunoassays comprising a combination of intimin and VTEC LPS would provide evidence of infection when VTEC cannot be isolated from patients.

References