Use of a Monoclonal Antibody against an Escherichia coli O26 Surface Protein for Detection of Enteropathogenic and Enterohemorrhagic Strains

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A monoclonal antibody (MAb) was obtained from a mouse immunized with solubilized outer membrane proteins extracted from a bovine enterohemorrhagic strain of Escherichia coli (EHEC), O26. The MAb produced a strong immunoblot reaction at approximately 21 kDa for an O26 strain containing the intimin gene (eae) and verocytotoxin (VT) production (19). Another pathogenic group, the enteropathogenic E. coli (EPEC), is also characterized by causing a/e lesions but differs from EHEC in that it does not produce VTs. Strains from both these groups are important causes of human enteric diseases (29). EHEC strains have become prominent in recent years as causes of hemorrhagic enteritis and the hemolytic uremic syndrome. The main serogroup implicated in human disease caused by EHEC has been O157 (10), but other serogroups, in particular O26, O103, O111, and O128, have also been implicated in causing human disease (13, 22, 32).

EHEC and EPEC strains are also associated with enteric disease in cattle (5, 6, 8, 20, 21, 25, 27, 31, 33, 37). The significance of these pathogenic groups in bovine enteritis is probably underestimated, possibly because of a lack of awareness of their significance and a lack of appropriate assays for routine detection. The widespread presence of VT-producing E. coli strains in healthy cattle is also a complication (3, 8, 26, 35). Demonstration of VT in cultures from bovine enteritis is not sufficient to imply a causative association. The object of the present study was to produce monoclonal antibodies (MAbs) to EHEC surface adhesion antigens, and to investigate their diagnostic application for the detection of EHEC in animal and human enteric infections. Because of an association with both human and bovine diseases, an EHEC strain of serotype O26 was selected for investigation.

MATERIALS AND METHODS

Preparation of antigens. An outer membrane (OM) preparation of E. coli O26 strain 4276 was prepared by the standard sarcosine extraction method (11). This strain was isolated from a calf enteritis case in Northern Ireland and was characterized as intimin (encoded by gene eae) and VT positive. Briefly, washed cells from an overnight broth culture, suspended in 0.01 M Tris HCl—0.005 M EDTA buffer, pH 7.8, were disrupted by ultrasonication. After centrifugation at 15,000 × g for 30 min to remove intact cells, the supernatant was mixed with a quarter volume of 2% (wt/vol) sodium n-laurylsarcosine (Sigma) in Tris-EDTA buffer at room temperature for 30 min and then centrifuged at 300,000 g for 1 h. The resuspended pellet was reextracted with an equal volume of 2% sarcosine for 1 h at room temperature, pelleted, washed once in saline, and stored at −70°C.

Some of the washed OM was solubilized in a 6 M solution of the chaotropic agent guanidine thiocyanate (Sigma) in Tris-EDTA. Insoluble material was removed by ultracentrifugation, and the outer membrane protein (OMP) solution was dialyzed against 100 volumes of 6 M urea in Tris-EDTA buffer and stored at −70°C.

MAbs. A BALB/c mouse was immunized intraperitoneally with the solubilized OMP preparation of E. coli O26 strain 4276. Three inoculations of 100 μl, 50 μl, and 50 μl of OMP solution, each mixed with 50 μl of adjuvant (125 μg of Quil A per ml) (Superfos; DK-Vedbaek, Denmark), were given at 4-week intervals. Three days after the final inoculation, the mouse spleen cells were fused with the NSO myeloma cells at a ratio of 8:1 according to the protocol of Galfre and Milstein (12) with modifications by Teh and Wong (34). The resulting hybridomas were maintained in RPMI 1640 medium (Gibco, Paisley, United Kingdom), supplemented with 20% gamma-globulin-free horse serum (Gibco).

The cell culture fluids from actively growing hybridomas were initially screened by enzyme-linked immunosorbent assay (ELISA) in microtiter plate wells (Dynatech, McLean, Va.) coated with OM preparations of E. coli O26
TABLE 1. E. coli strains used for the preliminary testing of MAb
2F3 and 6G5

| Source       | Strain     | Pathotypea | Serotype | eae | VT1  | VT2  | 2F3 ELISA | 6G5 ELISA |
|--------------|------------|------------|----------|-----|------|------|-----------|-----------|
| Bovine       | 4276       | EHEC       | O26      | +   | +    | +    | +         | +         |
| Bovine       | 237        | EHEC       | O26      | +   | +    | +    | +         | -         |
| Bovine       | 4618       | EPE    | O26      | +   | +    | -    | +         | -         |
| Human        | 1045       | ETEC      | O26      | -   | -    | -    | -         | -         |
| Bovine       | E7         | NTEC      | O15      | -   | -    | +    | -         | -         |
| Bovine       | S306       | NTEC      | UT      | -   | -    | -    | -         | -         |
| Bovine       | S1378      | NTEC      | UT      | -   | -    | -    | -         | -         |
| Bovine       | S784       | EHEC      | O157     | +   | +    | -    | -         | -         |
| Bovine       | 3680       | EPE    | O157     | +   | +    | -    | +         | +         |
| Bovine       | S951       | EHEC      | O157     | +   | +    | -    | -         | -         |
| Bovine       | 108        | VTEC      | O117     | -   | -    | +    | +         | +         |
| Bovine       | 286        | ETEC      | O141     | -   | -    | +    | +         | +         |
| Bovine       | 335        | ETEC      | O139     | -   | -    | +    | +         | +         |
| Bovine       | 413        | VTEC      | O103     | -   | -    | +    | +         | +         |
| Porcine      | 2353       | ETEC      | O149     | -   | -    | -    | -         | -         |
| Lapine       | B10        | EPE   | O103     | +   | +    | -    | +         | -         |
| Lapine       | E22        | VTEC      | O103     | +   | +    | -    | +         | +         |
| Human        | H217       | VTEC      | O146     | +   | +    | -    | +         | +         |

a ET EC, enterotoxigenic E. coli; NTEC, nectrotoxigenic E. coli; VTEC, vero-
cytotoxic E. coli.

RESULTS

MAb-based sandwich ELISA. Nine of the 430 hybridomas were selected as being reactive to OM preparations of E. coli strain 4276 and nonreactive to strain 1045. Three stable clones were derived from these, one of which was no longer reactive with strain 4276. Ascites fluid was prepared with the remaining two lines, MAbs 2F3 and 6G5, and used to prepare capture and biotinylated MAb reagents for use in sandwich ELISAs. Strain 4276 was used to optimize the assays.

Table 1 summarizes the sandwich ELISA results obtained with the collection of E. coli strains initially examined. The MAb 6G5 sandwich ELISA reacted only with the strain used to immunize the mouse for the hybridoma fusion. The MAb 2F3 sandwich ELISA reacted positively with only three O26 strains containing eae and/or the VT virulence factors. Neither of the assays reacted with Salmonella arizonae, Salmonella kentucky, Enterobacter spp., Klebsiella pneumoniae, Shigella flexneri, Pseudomonas fluorescens, Pseudomonas putida, Hafnia alvei, Serratia spp., Proteus vulgaris, Erwinia spp., Serratia liquefaciens, and Citrobacter freundii.

The sensitivity of detection for the MAb 2F3 sandwich ELISA for strain 4276 was 10^9 CFU/ml.

Test samples. The MAb 2F3 sandwich ELISA was used to screen various groups of E. coli strains. Table 2 summarizes the characteristics of 46 ELISA-positive and 42 ELISA-negative strains; these were largely from a collection of 216 strains isolated from animal enteritis cases in Northern Ireland but included two ELISA-positive bovine O111 strains from Belgium, 10 ELISA-negative Northern Ireland bovine O157 strains, and ELISA-negative O118 (n = 5), O5 (n = 2) O111 (n = 1), and O20 (n = 2) strains from Belgian cattle. The majority of the ELISA-positive strains were O26, although small numbers of other O-serotyopes were evident. In addition, the majority of the ELISA-positive strains were either eae or eae and VT positive, with low numbers of eae- and VT-negative strains or strains only positive for VT. All of the O18 ELISA-negative and the majority of O26 ELISA-negative strains were eae and VT negative, whereas both the ELISA-negative O111 strains were eae and VT positive. Included in Table 2 are serotypes recognized as important causes of bovine enteritis: O5, O20, O111, and O118, and bovine isolates of O157, all of which were positive for eae and/or VT.

Table 3 summarizes the ELISA results obtained with the remaining Belgian bovine strains examined, none of which was serotyped, but all of which were characterized for the presence from mixed agar cultures, demonstrating a positive ELISA reaction, were purified and tested individually. Purified, ELISA-positive reactants were O-serotyped and tested for the presence of eae and VT as above.

PCR. All of the ELISA-positive, and some of the ELISA-negative, strains were tested for eae by PCR. A proportion of these were similarly tested for the gene encoding VT. The primers used in the procedure differed in the Northern Irish (23) and the Belgian (7) laboratories.

Serogrouping. All E. coli isolates obtained in Northern Ireland were O-typed by slide agglutination (30), by using a collection of 74 antisera, raised mainly against strains of veterinary importance.

Immunoblotting. Polyclonal and MAb-based sandwich ELISA was carried out on whole-cell preparations of E. coli K-12 (eae-positive and eae-negative strains) and O157 (eae-positive strains). NuPAGE 4 to 12% gels (Novex, San Diego, Calif.) were used, in accordance with manufacturer’s instructions. The bands were transferred onto nitrocellulose by overnight blotting at 30 mA. The nitrocellulose was blocked with 5% (wt/vol) bovine serum albumin (Oxoid) in PBS buffer (0.1 M PBS with 0.5% Tween 80 [vol/vol], 2 g of NaCl [wt/vol], 0.001 M EDTA), pH 7.2. Following incubation with biotinylated MAb dilutions at 37°C for 1 h, the nitrocellulose strips were washed in PBS buffer before the addition of streptavidin peroxidase for a further 1 h. After another wash, the peroxidase substrate, 0.5 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma) per ml in 0.02 M Tris-HCl buffer, pH 7.2, with 0.3 μl of H2O2 (30% solution) per ml (vol/vol), was added. After incubation at room temperature for 10 min, the strips were washed with distilled water, which stopped any further reaction.

Screening of E. coli by sandwich ELISA. The MAb 2F3 sandwich ELISA was used to screen various groups of E. coli strains. Table 2 summarizes the characteristics of 46 ELISA-positive and 42 ELISA-negative strains; these were largely from a collection of 216 strains isolated from animal enteritis cases in Northern Ireland but included two ELISA-positive bovine O111 strains from Belgium, 10 ELISA-negative Northern Ireland bovine O157 strains, and ELISA-negative O118 (n = 5), O5 (n = 2) O111 (n = 1), and O20 (n = 2) strains from Belgian cattle. The majority of the ELISA-positive strains were O26, although small numbers of other O-serotypes were evident. In addition, the majority of the ELISA-positive strains were either eae or eae and VT positive, with low numbers of eae- and VT-negative strains or strains only positive for VT. All of the O18 ELISA-negative and the majority of O26 ELISA-negative strains were eae and VT negative, whereas both the ELISA-negative O111 strains were eae and VT positive. Included in Table 2 are serotypes recognized as important causes of bovine enteritis: O5, O20, O111, and O118, and bovine isolates of O157, all of which were positive for eae and/or VT.

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of eae, and some for the presence of VT. Twenty-one of the 56 strains isolated from 6- to 10-week-old calves that had died with enteritis were ELISA-positive, and 35 were ELISA negative; both groups of these strains were entirely eae positive, with or without VT. Twenty-three out of 67 E. coli strains isolated from 0- to 10-week-old calves that had died of enteritis were ELISA positive, and 35 were ELISA negative. Both groups of these strains were entirely eae positive, with enteritis were ELISA positive, and 35 were ELISA negative. Twenty-one of the 56 strains isolated from 6- to 10-week-old calves that had died with enteritis were ELISA positive, and 35 were ELISA negative; both groups of these strains were entirely eae positive, with or without VT. Twenty-three out of 67 E. coli strains isolated from 0- to 10-week-old calves that had died of enteritis were ELISA positive, and 35 were ELISA negative. Both groups of these strains were entirely eae positive, with enteritis were ELISA positive, and 35 were ELISA negative. Twenty-one of the 56 strains isolated from 6- to 10-week-old calves that had died with enteritis were ELISA positive, and 35 were ELISA negative; both groups of these strains were entirely eae positive, with or without VT.

**Table 2. Characterization of the E. coli strains demonstrating positive and negative reactions in the MAb 2F3 sandwich ELISA**

| Strain origin | ELISA | Total no. of isolates | No. eae positive | No. VT positive | No. eae and VT positive | No. eae and VT negative |
|---------------|-------|-----------------------|------------------|-----------------|------------------------|-------------------------|
| O26           | +     | 33                    | 10               | 3               | 18                     | 2                       |
|               | −     | 10                    | 1                | 0               | 0                      | 8                       |
| O18           | +     | 3                     | 1                | 1               | 1                      | 1                       |
|               | −     | 11                    | 0                | 0               | 0                      | 11                      |
| O111          | +     | 6                     | 3                | 0               | 3                      | 0                       |
|               | −     | 2                     | 0                | 0               | 2                      | 0                       |
| O157          | +     | 0                     | 0                | 0               | 0                      | 0                       |
|               | −     | 10                    | 1                | 0               | 9                      | 0                       |
| Others        | +     | 4                      | 1                | 1               | 1                      | 1                       |
|               | −     | 9                      | 2                | 5               | 2                      | 0                       |

* One each of O73, O113, O69, and UT.

**Table 3. Characterization of Belgian ELISA-positive and -negative E. coli strains**

| Strain origin          | ELISA | Total no. of isolates | No. eae positive | No. VT positive | No. eae and VT positive | No. eae and VT negative |
|------------------------|-------|-----------------------|------------------|-----------------|------------------------|-------------------------|
| Calves, 0–10 wk old, dead from enteritis | +     | 21                    | 12               | 0               | 9                      | 0                       |
|                        | −     | 35                    | 19               | 0               | 16                     | 0                       |
| Calves, 6 mo old, normal | +     | 23                    | 16               | 1               | 6                      | 0                       |
|                        | −     | 44                    | 8                | 15              | 10                     | 11                      |
| Calves, 2–8 wk old, with enteritis | +     | 115                   | 115              | NT              | NT                     | NT                      |
|                        | −     | 78                    | 78               | NT              | NT                     | 0                       |

**DISCUSSION**

The MAb 2F3 produced in this study demonstrated a high level of specificity for a group of E. coli strains, in particular, strains of serotype O26, with the potential to express the EHEC and EPEC virulence factors of eae and VT. eae is the gene for the expression of intimin, which is regarded as a significant virulence factor in both EHEC and EPEC strains. If it is assumed that all strains with eae are potentially pathogenic, the application of MAb 2F3 in a sandwich ELISA format enables the rapid detection of a group of pathogenic strains from within these groups.

The identity of the antigen detected by MAb 2F3 is not clear. Immunoblotting demonstrated a strong reaction at 21 kDa with only the eae- and VT-positive O26 antigen used. From the molecular weight and surface presence of this protein, it is possible that it is fimbriae (14) or the recently described EspA protein (18). The former is induced by the loss of antigen from six strains following storage, possibly from plasmid loss. Fimbriae implicated in early host cell adhesion of EPEC strains, named the bundle forming pili (bfp), have been defined as plasmid located (14). Giron et al. (15), using a molecular probe, demonstrated that bfp were only present in the EPEC strains of the human pathogenic E. coli strains that were examined. China et al. (9) failed to find bfp in animal EPEC or EHEC strains by using this human EPEC probe. Wieler et al. (36) demonstrated a significant increase in cell attachment of bovine EHEC O118 strains on fetal calf lung cells (90.5%) compared with human HEP-2 cells (52.4%) by using the fluorescent actin staining test (17). These studies indicate differences in adhesins between EPEC and EHEC.
TABLE 4. Results obtained with the MAb 2F3 sandwich ELISA on field isolates from enteritis cases

| Strain origin | No. tested | No. ELISA positive (%) |
|---------------|------------|------------------------|
| Bovine        | 366        | 41 (11.2)              |
| Porcine       | 42         | 3 (7.1)                |
| Ovine         | 40         | 3 (7.5)                |
| Canine        | 8          | 1 (12.5)               |
| Avian         | 31         | 1 (3)                  |
| Other         | 8          | 0 (0)                  |
| Human         | 490        | 44 (9.2)               |
| Total         | 985        | 93 (9.4)               |

It is possible that the antigen detected in the present study is an alternative to bfp for preliminary cell attachment. If this is confirmed, since it was demonstrated in both human- and animal-isolated strains, it must be concluded that either there is a common host receptor or that these strains possess more than a single host cell attachment mechanism.

The loss of the antigen detected by the ELISA in six strains which did not possess either the eae or the VT factor and its presence in a small number of VT-positive and eae-negative isolates demonstrate its occurrence in non-EPEC and non-EHEC strains. It can be speculated that the presence of eae and/or VT provides some plasmid stability, but whether these strains are of any pathological significance is unknown and requires further investigation. Nonpathogenic strains, such as many VT-producing strains, can express a virulence factor(s). It is recognized that virulence is the result of a combination of factors which, individually, have limited pathogenic effect. Apart from experimental infection, the significance of these factors in bacterial strains is determined by their presence in combination with other factors, and by their more-common occurrence in strains isolated from diseased animals or humans. The association of the vast majority of E. coli strains that reacted with MAb 2F3 to the presence of the gene for intimin, which is regarded as a virulence factor of notable significance, is a strong indication of the importance of the antigen to which it reacts.

The association of MAb 2F3 with eae was in the presence or absence of VT factors. Because MAb 2F3 associated with eae in the presence and absence of VT factors, the assay developed could not distinguish between EHEC and EPEC strains. This result indicates a close relationship between the strains detected from the two pathogroups. Whether the virulence differences of these groups are associated with the presence or absence of VT requires further investigation.

A number of eae-positive strains with or without VT did not react with MAb 2F3. These include a number of strains of recognized bovine (O118 and O5), rabbit (O103), and human (O157) pathogenicity. In addition, of the 10 eae- or eae- and VT-positive O111 strains examined, only eight were ELISA reactive. If the antigen targeted by this MAb is confirmed as making a significant contribution to the virulence of EHEC and EPEC strains, it could be concluded that these nonreactive strains possess antigenic variations of this factor.

The high prevalence of O26 strains amongst the positive reactants to this MAb indicates the probable significance of this serotype in animal enteritis in Northern Ireland. A number of O26 strains were also present in the ELISA-negative group, the majority of these being eae and VT negative. This indicates that other pathogroups of this serotype are of probable significance in this condition.

Although only a small number of strains from human diarrhea were tested, the demonstration, by ELISA, of a common antigen in bovine and human isolates could indicate a zoonotic risk of bovine strains to humans. The commonality of the MAb-detected antigen was also demonstrated in the results obtained with isolates from the field survey of human and animal enteritis (Table 4). The fact that strains from the same serotypes have been implicated in both bovine and human diseases (O26, O111, and O18) also supports these findings. The presence of a high percentage of EHEC and EPEC strains in both ELISA-positive and -negative groups isolated from healthy calves sampled at an abattoir (Table 4) indicates a significant potential for infection of susceptible cattle and for zoonotic transfer to humans.

The results of the survey conducted with nearly 1,000 animal and human enteric isolates demonstrated a significant presence of strains expressing the targeted antigen (Table 4), in particular from bovine, human, ovine, and porcine samples. Although only five EHEC-EPEC strains were purified from these samples by the limited method employed, the presence of a virulence-associated antigen was demonstrated in a high proportion of the mixed cultures. This finding indicates a significant pathogenic role of EHEC and EPEC strains in both human and animal enteric diseases and highlights the diagnostic potential of the assay developed. Further studies to develop MAbs to surface antigens of the eae-positive strains that were nonreactive to the O26 MAbs in this study would clarify the significance of the antigens in terms of virulence and virulent-strain detection.

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