Adhesive Properties Associated with the Vir Plasmid: A Transmissible Pathogenic Characteristic Associated with Strains of Invasive *Escherichia coli*

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*Escherichia coli* strain S5 (O15:K⁺:H21) isolated from a septicaemic lamb and previously shown to possess a virulence plasmid, Vir, attached *in vitro* to calf epithelial tissue from the ileum, oesophagus and trachea in the presence of 0·5% (w/v) D-mannose. The Vir⁺ recombinant strains 711v and H209av, which had received the Vir plasmid(s) from strain S5, also attached to these epithelia but the parent strains 711 and H209a without the Vir plasmid were non-adhesive. The attachment of the Vir⁺ strain 711v to intestinal brush borders was inhibited by antiserum to live Vir⁺ strain H209av but not by antiserum to strain H209a lacking Vir. No adherence occurred with Vir⁺ organisms grown at 18 °C or after heating at 65 °C. Adhesion was unaffected by 0·5% (w/v) formaldehyde. Glucosamine, mannosamine, their N-acetyl derivatives and wheat germ lectin each inhibited attachment of Vir⁺ strain 711v to brush border epithelia.

INTRODUCTION

Enteropathogenic strains of *Escherichia coli* causing neonatal diarrhoea are usually confined to the alimentary tract but bacteraemic strains of this organism invade the host tissues causing 'coli-septicaemia' (Sojka, 1971). Smith (1974) identified two transmissible plasmids, ColV and Vir, which appear independently in certain strains of invasive *E. coli*. ColV synthesis is a common feature in septicaemic *E. coli* from calves but ColV also occurs in isolates from man, lambs and pigs. All the *E. coli* strains expressing the Vir phenotype have been isolated from calves and lambs affected with *E. coli* septicaemia (Smith, 1974; Lopez-Alvarez & Gyles, 1980). The ColV plasmid appears to increase the resistance of the pathogen to the host defense mechanisms while the Vir⁺ phenotype is associated with the synthesis of toxin(s) and surface antigen(s) (Smith, 1974; Lopez-Alvarez & Gyles, 1980). The Vir toxin appears to be very similar to the 'chicken lethal toxin' described previously by Truscott (1973) but the properties of the Vir surface antigen have not been described. We report that in common with the plasmid-controlled surface antigen K88 (Ørskov & Ørskov, 1966), K99 (Ørskov et al., 1975) CFA/I and CFA/II (Evans et al., 1975; Evans & Evans, 1978) found on enteropathogenic *E. coli*, the presence of the Vir plasmid in invasive *E. coli* confers adhesive properties *in vitro*.

METHODS

*Bacteria.* All the strains used in this study were kindly supplied by Dr H. Williams Smith. Details of these strains are summarized in Table 1. Unless stated otherwise all bacteria were cultured on sheep blood agar at 37 °C.

*Antiserum.* Antiserum was prepared in rabbits by a total of five intravenous injections of live organisms at 3 d intervals. Blood was collected 7 d after the last injection (Sojka, 1965). To prepare absorbed factor Vir antiserum, antiserum to the Vir⁺ strain 711v was absorbed with live Vir⁻ organisms of strain 711 at 37 °C overnight and the supernatant collected by centrifugation. Sodium azide was added to a final concentration of 10 mM.

*Isolation of epithelial cells.* Epithelial tissue was prepared from healthy calves (1 to 3 weeks old). The ileum brush border epithelia were isolated using the technique described by Sellwood *et al.* (1975).
Table 1. Strains examined in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Derivation</th>
<th>Serotype</th>
<th>Vir-phenotype</th>
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<tbody>
<tr>
<td>S5</td>
<td>Vir donor strain isolated from blood of lamb with fatal bacteraemia</td>
<td>O15:K+:H21</td>
<td>Vir+</td>
</tr>
<tr>
<td>H209a</td>
<td>Spontaneous K− derivative of H209, isolated from faeces of healthy human</td>
<td>O9:K31−:H14</td>
<td>Vir−</td>
</tr>
<tr>
<td>H209av</td>
<td>Vir plasmid carrying recombinant from S5 × H209a mating</td>
<td>O9:K−:H14</td>
<td>Vir+</td>
</tr>
<tr>
<td>711</td>
<td>K12 strain</td>
<td>R</td>
<td>Vir−</td>
</tr>
<tr>
<td>711v</td>
<td>Vir plasmid carrying recombinant from S5 × 711 mating</td>
<td>R</td>
<td>Vir+</td>
</tr>
</tbody>
</table>

The trachea and oesophagus were opened by cutting along their lengths under cold 100 mM-phosphate buffer pH 7.2. The mucosa was washed using a jet of buffer from a pipette, rinsed and placed in fresh buffer. A glass microscope slide was then used to scrape off epithelium fragments which were collected by pipette.

Adhesion tests. Epithelial cell suspensions (100 μl) were incubated at 37 °C for 60 min with equal volumes of bacteria. The final concentration of epithelial cells was approximately 10⁶ ml⁻¹ and the bacteria were at a final concentration of approximately 10⁹ ml⁻¹. The number of bacteria adhering to individual epithelial cells was determined using interference microscopy. Drops from the reaction mixture were examined directly under coverslips on glass slides. Aggregates or sheets of tissue were disregarded. The first 20 well defined epithelial cells were examined in each experiment and the results from experiments on at least two different days were combined to determine the mean number of bacteria adhering per epithelial cell. D-Mannose at 0.5% (w/v) was present in every experiment, except the sugar studies, to prevent attachment by type 1 fimbriae. There were no pre-existing bacteria on epithelial cells. With the exception of squamous epithelium, adhesion was easy to quantitate as the bacteria were well distributed on the tissue and both bacteria and epithelial cells were clearly visible. When the effect of sugars (100 mM) or lectins (final concentration 50 μg ml⁻¹) was examined the reagents were added with the bacteria. The significance (P < 0.05) between attachment of bacteria to brush border epithelial cells in the presence of sugars compared with saline and D-mannose was determined by Student’s t test. In the washing experiment the mixture of bacteria and brush borders were centrifuged at 200 g for 10 min after the 60 min incubation. The pellet was resuspended using a rotary mixer and the procedure was then repeated. The effect of formaldehyde on adhesion was investigated using the conditions described by Duguid et al. (1979) for the differentiation of fimbrial haemagglutination. The bacteria were washed free of formaldehyde before examination. Serum inhibition of bacterial attachment was investigated by performing the adhesion experiments in the presence of heterologous antisera.

Haemagglutination. Direct bacterial haemagglutination of red cells from cattle, horse, man (blood group A), pig and sheep was examined using 200 μl volumes of 3% (v/v) red cells and an equal volume of a dense bacterial suspension. D-Mannose was present at a concentration of 0.5% (w/v) to prevent haemagglutination by type 1 fimbriae. Microtitre plates were incubated at 4 °C for 2 h.

Electron microscopy. Cultures of bacteria grown on blood agar slopes for 18 h were gently washed off with distilled water and examined for fimbriae by negative staining with 0-1% (w/v) phosphotungstic acid using a Philips EM300 electron microscope.

For scanning electron microscopy adhesion tests were performed as described but suspensions of bacteria and tissue were washed on filters. The filter was removed from filters with phosphate buffer, fixed in 3% (w/v) glutaraldehyde (in 100 mM-phosphate buffer, pH 7.2) for 2 h, washed and post fixed in osmium tetroxide (1%, w/v). After dehydration through graded alcohols and finally acetone, samples were critically point dried in CO₂, sputter coated with gold and examined by scanning electron microscopy by Dr B. Brooker at the National Institute for Research in Dairying, Reading.

RESULTS

All the Vir+ strains (S5, 711v and H209av) agglutinated in the absorbed Vir antiserum when the bacteria were grown at 37 °C. The same sera did not agglutinate the Vir− strains 711 or H209a or any of the strains grown at 18 °C.

Haemagglutination

None of the cultures, irrespective of the presence or absence of the Vir plasmid, haemagglutinated red cells from horse, man, pig or sheep at 4 °C in the presence of mannose. Weak mannose-resistant haemagglutination of freshly drawn bovine red cells was observed with dense
Organisms of the Vir donor strain S5 and the Vir+ strains 711v and H209av attached to brush border epithelia from calf ileum, to squamous epithelia from the oesophagus and to ciliated epithelia from the trachea in the presence of mannose (Table 2 and Fig. 1). The Vir- strains 711 and H209a did not attach to these tissues.

Attachment to calf ileum brush borders was sensitive to heat and did not occur if the bacteria were cultured at 18 °C (Table 3). However, the adhesion was unaffected by formalin. Furthermore, once the bacteria were attached, they were still present after two cycles of washing.

The effect of sugars on the attachment of the Vir+ strain 711v to calf brush borders is summarized in Table 4. With the exception of galactose and its derivatives, all the sugars tested significantly reduced attachment ($P < 0.01$) at a final concentration of 100 mM. However, in order to block any activity due to type 1 fimbriae, mannose was included routinely in all experiments other than those designed to investigate the effect of free sugars on adhesion. The results were therefore also compared to the sample containing mannose. Glucosamine, mannosamine and their N-acetyl derivatives reduced attachment significantly more than mannose. Galactose and its derivatives did not inhibit as strongly as mannose whereas glucose inhibited to the same degree as mannose.

Wheat germ lectin, which is specific for N-acetyl glucosamine, blocked attachment (90% inhibition) but castor bean lectin which is specific for N-acetyl galactosamine and lectins from asparagus pea (specific for L-glucose) and garden pea (specific for glucose and mannose) had little effect (each 10% inhibition). These experiments were performed in the presence of mannose.
Fig. 1. Scanning electron micrograph of *in vitro* preparation of calf ciliated epithelia after incubation with (a) Vir− 711 bacteria and (b) Vir+ 711v bacteria. The bar markers represent 1 μm.
Table 4. Effect of sugars on the in vitro attachment of strain 711v (Vir+) to calf brush border epithelia

<table>
<thead>
<tr>
<th>Sugar (100 mm)</th>
<th>No. of brush border cells counted</th>
<th>Mean no. (± s.d.) of bacteria per border</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>60</td>
<td>11.75 ± 3.97</td>
</tr>
<tr>
<td>Glucose</td>
<td>40</td>
<td>6.65 ± 1.21*</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>40</td>
<td>4.4 ± 3.60*†</td>
</tr>
<tr>
<td>N-Acetyl glucosamine</td>
<td>60</td>
<td>0.28 ± 0.73*†</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>40</td>
<td>6.8 ± 1.87*‡</td>
</tr>
<tr>
<td>D-Mannosamine</td>
<td>60</td>
<td>2.65 ± 2.56*†</td>
</tr>
<tr>
<td>N-Acetyl mannosamine</td>
<td>60</td>
<td>0.45 ± 0.83*†</td>
</tr>
<tr>
<td>Galactose</td>
<td>40</td>
<td>10.1 ± 2.40</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>40</td>
<td>9.8 ± 2.73</td>
</tr>
<tr>
<td>N-Acetyl galactosamine</td>
<td>40</td>
<td>9.77 ± 3.46</td>
</tr>
</tbody>
</table>

* Significant reduction (P < 0.01) compared to saline control.
† Significant reduction (P < 0.01) compared to D-mannose.

In serum inhibition experiments there were small groups of agglutinated Vir+ organisms of the strain 711v in the presence of antiserum to both the Vir+ strain H209av and the Vir- strain H209a at final dilutions of 1 in 200. However, when the sera were diluted to 1 in 400 there was no bacterial agglutination but the antiserum to the Vir+ strain H209av, at this dilution, reduced attachment of the Vir+ strain 711v by 97.2%, while the antiserum to the Vir- strain H209a caused a reduction of 10-2% relative to the control which contained normal rabbit serum diluted 1 in 400. Mannose was present in each of these reaction mixtures.

Electron microscopy

Negative staining revealed numerous fimbriae on all the strains irrespective of the presence or absence of the Vir plasmid.

The scanning electron micrographs of ciliated epithelia from the trachea illustrates the attachment of the Vir+ organisms of the strain 711v after washing on a membrane filter (Fig. 1). Attachment appeared to be restricted to the cilia.

DISCUSSION

Early in the pathogenesis of E. coli septicaemia invasive organisms must come into intimate contact with a mucosal surface, probably the mucosa of the intestinal or upper respiratory tract. To achieve this the pathogen has to overcome the mutual repulsion between its own surface and the hosts. Escherichia coli enteropathogenic for piglets produce K88 (Ørskov et al., 1961), K99 (Moon et al., 1977) or 987P (Nagy et al., 1977); calf and lamb enteropathogens produce K99 (Smith & Linggood, 1972; Ørskov et al., 1975), while E. coli enteropathogenic for man produce CFA/I (Evans et al., 1975) or CFA/II (Evans & Evans, 1978) antigens. These are all fimbrial-like structures associated with the attachment of the pathogen to the brush border epithelium of the small intestine.

The present study demonstrated that strain S5, the Vir donor and the Vir+ recombinant strains 711v and H209av attached in vitro to the three types of mucosal epithelium examined from calves. The Vir- strains 711 and H209a did not attach to epithelia, and differed from the recombinants only in the possession of the Vir plasmid, it appears that the adhesive properties are conferred by the presence of a transmissible plasmid. This was confirmed by the significant reduction of attachment of the Vir+ strain 711v in the presence of antiserum to the Vir+ strain H209av but not with antiserum to the Vir- strain H209a.

Mannose resistant and eluting (MRE) haemagglutinating activity in enterobacteria is generally associated with adhesiveness for epithelia (Duguid et al., 1979). MRE haemagglutinating activity could not be detected with the Vir+ 711v organisms tested against red cells from five species of animal. However, the Vir+ recombinant strain H209av and the Vir donor strain S5
exhibited weak activity with bovine red cells in the presence of mannose. Thus, the Vir phenotype may confer haemagglutinating activity for bovine red cells and the differences between the two Vir+ recombinant strains may be quantitative. The K88, K99, CFA/I and CFA/II adhesins are each associated with MRE haemagglutinating activity. Organisms producing 987P do not haemagglutinate guinea-pig red cells (Nagy et al., 1977) but their reaction with other red cells has not been reported.

In common with the enteropathogenic E. coli producing K88 or K99 (Burrows et al., 1976), the adhesion of Vir+ organisms to intestinal brush borders occurred in the presence of D-mannose and the activity was lost when the bacteria were heated at 65 °C. K88 and K99 are not detectable and adhesion does not occur, when organisms capable of producing these antigens are grown at 18 °C (Morris et al., 1980). Similarly, when the Vir+ organisms were cultured at 18 °C adhesion did not occur and the Vir surface antigen could not be detected. In contrast to E. coli producing K88 or K99 (Morris & Thorns, unpublished results), the Vir+ organisms adhered after treatment with formaldehyde.

Type 1 fimbriae are mannose-sensitive adhesins. They are produced at 20 °C and are resistant to heating at 65 °C and to formaldehyde treatment (Duguid et al., 1979). The mannose-resistant adhesin associated with the Vir plasmid therefore shares properties with both mannose-resistant and mannose-sensitive adhesins. Although type 1 fimbriae are usually produced in static liquid cultures grown serially for 24 h or longer, they may be produced to a limited extent by cultures grown on agar (Duguid & Old, 1980). D-Mannose had a significant effect on the adhesion of the Vir+ organism 71lv, but this effect was also observed with glucose. The action of D-mannose on the activity of type 1 fimbriae is highly specific (Duguid & Old, 1980). Nevertheless, because it is possible that type 1 fimbriae are produced by Vir+ organisms, D-mannose was used routinely to avoid possible confusion caused by the presence of type 1 adhesins.

The nature of the factor(s) which mediates the attachment of Vir+ organisms to epithelial cells has not been defined. Both the Vir+ and their Vir- counterparts produced fimbriae. Lopez-Alverez & Gyles (1980) reported that the transmission of a Vir plasmid into a recipient which did not produce fimbriae produced a recombinant organism which did produce these structures. The susceptibility of the Vir adhesin to heating suggests that the Vir adhesin may be protein and the presence of adherent bacteria on brush borders after two cycles of washing demonstrates a strong association. Furthermore, the reduction of adhesion in the presence of only two of the N-acetyl amino sugars examined together with the blocking of attachment by wheat germ lectin but not by the other lectins examined, suggests that the interaction involves specific determinants.

Adherence may be an initial step in the pathogenesis of coli-septicaemia. Unlike coliform mastitis, coli-septicaemia is associated with E. coli from relatively few serological groups and it would be of interest to determine whether representatives from these groups also exhibit adhesiveness in vitro, and, if so, whether adhesion occurs in vivo.

REFERENCES


