1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) have been found to be associated with acute diarrhoea in animals and humans for more than a decade [1]. Pilus-like surface antigens on porcine strains (K 88 antigen) and on strains from calves and lambs (K 99 antigen) [1] and the colonization factor antigens (CFA/I and CFA/II) [2,3] on human strains have been implicated as the structures that facilitate adherence to the epithelial cells of the small intestine of pigs, calves and lambs and humans respectively [4]. Colonization of the gut may then occur which is regarded as the prerequisite to bacterial diarrhoea. However, it was not until recently that studies on the nature of the cell receptors for these colonizing factors or adhesins started [4,6]. Recent observations indicate that the intestinal receptor for K 88 antigen may be a glycolipid [6]. It was also found that certain glycopeptide(s) inhibit binding of K 88 to erythrocytes [5]. The difficulties involved in obtaining intestinal brush border preparations from different animal species encouraged us to study the erythrocyte receptor that is responsible for haemagglutination (HA) of CFA/I and K 99 strains. In contrast to the HA caused by type I (common type) pilus which is sensitive to inhibition by D-mannose (MSHA) [7], K 88 and K 99 antigens in animal ETEC strains and CFA/I and CFA/II in human ETEC strains all cause D-mannose resistant HA (MRHA) reaction.

2. Materials and Methods

2.1. Bacteria

The various *E. coli* strains studied are described with respect to origin, serotype, and/or enterotoxicity in recent communications [8,9]. The K 99 strains were kindly supplied by Dr. C.J. Smyth (Department of Bacteriology and Epizootology, Biomedicum, Box 583, Uppsala, Sweden). These strains were originally from Dr. O. Söderlind (National Veterinary Institute, S-750 07 Uppsala Sweden). One non-enterotoxigenic *E. coli* was obtained from a patient with urinary tract infection at the Karolinska Hospital in Stockholm (Sweden) and was kindly supplied by Dr. A. Ljungh [10]. The characteristics of the strains are summarized in Table 1. Strains were stored in Trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Ma.) containing 15% (w/v) glycerol at −70°C and/or passaged on heart infusion deep agar at 3 month intervals.

2.2. Growth conditions

K 99-positive strains were grown on improved Minca medium [11] and all human ETEC and non-ETEC strains were grown on improved CFA agar medium [2]. All cultures were incubated at 37°C for 18 h.

2.3. Haemagglutination test

ETEC and non-ETEC bacteria showing MRHA for different erythrocyte species were suspended in physiological saline (pH 6.9). HA tests and HA inhibition tests were performed in glass depression
TABLE 1
Characteristics of *Escherichia coli* examined for haemagglutination and haemagglutination inhibition reactions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serogroup</th>
<th>MRHA with <em>a</em> erythrocyte</th>
<th>Antigen</th>
<th>Enterotoxin <em>b</em> production</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E 1628-15</td>
<td>078 : KN : NM <em>c</em></td>
<td>human</td>
<td>CFA/I</td>
<td>LT*-ST*+</td>
<td>human diarrhoea</td>
</tr>
<tr>
<td>E 54e-14</td>
<td>078 : KN : M</td>
<td>human</td>
<td>CFA/I</td>
<td>LT*-ST*+</td>
<td>human diarrhoea</td>
</tr>
<tr>
<td>2016-10</td>
<td>078 : ND : M</td>
<td>human</td>
<td>CFA/I</td>
<td>LT*-ST*+</td>
<td>human diarrhoea</td>
</tr>
<tr>
<td>H 10407</td>
<td>078 : K80 : H11</td>
<td>human</td>
<td>CFA/I</td>
<td>LT*-ST*+</td>
<td>human diarrhoea</td>
</tr>
<tr>
<td>Bd 854/75</td>
<td>0101, NM</td>
<td>sheep</td>
<td>K 99</td>
<td>LT*-ST*+</td>
<td>pig diarrhoea</td>
</tr>
<tr>
<td>B 539/76</td>
<td>0112ab, K, H8</td>
<td>sheep</td>
<td>K 99</td>
<td>LT*-ST*+</td>
<td>pig diarrhoea</td>
</tr>
<tr>
<td>Bd 2068/75</td>
<td>0101, K, NM</td>
<td>sheep</td>
<td>K 99</td>
<td>LT*-ST*+</td>
<td>pig diarrhoea</td>
</tr>
<tr>
<td>431 <em>d</em></td>
<td>0101K30 (A) : NM</td>
<td>sheep</td>
<td>K 99</td>
<td>LT*-ST*+</td>
<td>pig diarrhoea</td>
</tr>
<tr>
<td>C 921b-1</td>
<td>06 : K15 : M</td>
<td>bovine</td>
<td>CFA/I</td>
<td>LT*-ST*+</td>
<td>human diarrhoea</td>
</tr>
<tr>
<td>E 80</td>
<td>06 : K15 : H-</td>
<td>bovine</td>
<td>CFA/I</td>
<td>LT*-ST*+</td>
<td>human diarrhoea</td>
</tr>
<tr>
<td>2683a</td>
<td>06 : KN : NM</td>
<td>human</td>
<td>CFA/I</td>
<td>LT*-ST*+</td>
<td>urinary tract infec-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K 99</td>
<td>tion (human)</td>
</tr>
</tbody>
</table>

*a* MRHA, Mannose-resistant haemagglutination reaction performed on glass depression slides.

*b* LT, heat-labile enterotoxin, production assayed in the CHO or adrenal cell test. ST, heat-stable enterotoxin, production assayed in suckling 3-day-old mouse test by the method of Gianella.

*c* M, motile; NM, non-motile.

*d* Kindly supplied by H.W. Moon, National Animal Disease Center, Ontario, Canada.

Bacterial suspensions 

Slides in the presence of D-mannose (0.5% w/v) [8]. Bacterial suspensions 

(10⁷—10⁹ cells/ml) were titrated against erythrocyte suspensions (3% v/v) to obtain a minimal bacterial haemagglutinating dose that is defined as the smallest amount of bacteria which gives visible HA reaction.

2.4. Haemagglutination inhibition (HI) experiments

(i) Inhibition with glycolipids: Bacterial suspensions were preincubated with the potential inhibitors (disialogangliosides, Sigma type II, and monosialo-gangliosides, Sigma type III) and purified GM₁, GM₂, GM₃ (10 mg/ml) or N-acetylneuraminic acid (NANA, 2.3 mg/ml, pH adjustment with 1 M NaOH to pH 7.0) and heparin (1000 I.U./ml equivalent to 5.8 mg/ml) for 5 min at 20°C before the erythrocyte suspension was added in the HA assay. Erythrocyte suspension (10 μl of 3% v/v) was added and HA reactions were read after further incubation at 20°C for 10 min.

(ii) Enzyme treatment: Erythrocyte suspensions (sheep, human and bovine) were incubated at 37°C for 30 min with trypsin (0.5 mg/ml), Pronase (1 mg/ml), or clostridial neuraminidase (1 I.U./ml) followed by addition of bacterial suspension in the HA assay (10⁷ cells/ml).

2.5. Enterotoxicity

Production of heat-labile (LT) enterotoxin was assayed in the adrenal Y1 cell test or the Chinese Hamster Ovary cell (CHO) test [12,13]. Heat-stable (ST) enterotoxin was detected in the suckling mouse test [14].

2.6. Chemicals

Commercial preparations of partially purified gangliosides were obtained from Sigma Chem. Co., St. Louis, Mo., U.S.A. (type II, batch No. 470-8080 and type III batch No. 750-8200). Neuraminidase from *Clostridium perfringens* (type VIII, batch No. 1080-8035) and NANA (type VII from human urine), trypsin (2 X cryst., type III), cerebrosides (type I and II, batch no. 440-82702 and batch No. 1160-8030) were also obtained from Sigma. Pronase was purchased from Merck AG, Darmstadt, Germany. Heparin was a kind gift from Vitrum, Stockholm, Sweden. Highly purified gangliosides (GM₁, GM₂ and GM₃) were kind
TABLE 2

Inhibition of K 99 and CFA/I haemagglutination of human erythrocytes

*Thin-layer chromatography patterns:* Gangliosides Type II (disialogangliosides) are slow migrating and Type III (monosialogangliosides) are fast migrating. Cerebrosides Types I and II are slow and fast migrating respectively (information was obtained from the manufacturer).

<table>
<thead>
<tr>
<th>Potential inhibitor</th>
<th>Reaction a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gangliosides Type II</td>
<td>-</td>
</tr>
<tr>
<td>Gangliosides Type III</td>
<td>+</td>
</tr>
<tr>
<td>Cerebrosides Type I</td>
<td>-</td>
</tr>
<tr>
<td>Cerebrosides Type II</td>
<td>-</td>
</tr>
<tr>
<td>GM₁</td>
<td>-</td>
</tr>
<tr>
<td>GM₂</td>
<td>+</td>
</tr>
<tr>
<td>GM₃</td>
<td>-</td>
</tr>
<tr>
<td>NANA</td>
<td>-</td>
</tr>
<tr>
<td>Heparin</td>
<td>-</td>
</tr>
</tbody>
</table>

a + indicates inhibition and – indicates no inhibition of haemagglutination.

gifts from Dr. S. Hammarsröm, Department of Chemistry, Karolinska Institutet, Stockholm.

3. Results

3.1. Haemagglutination inhibition with glycolipids

Preliminary HI experiments with crude commercial ganglioside preparations showed that monosialo-gangliosides (type III) and disialogangliosides (type II) inhibited the HA reaction with four K 99 and four CFA/I strains (Table 2), but cerebrosides, at the same concentration (10 mg/ml) did not inhibit. It should also be pointed out that strains possessing other surface antigens causing MRHA reactions (CFA/II, MRHA non-ETEC) did not show any inhibitable HA reactions with any of these crude gangliosides (Table 2).

Experiments with highly purified ganglioside preparations at different concentration in the incubation mixture showed that only GM₂ at high concentration (10 mg/ml), i.e., above the critical micellar concentration (cmc) of 7.5 \cdot 10^{-5} M was able to inhibit the MRHA of CFA/I and K 99 strains, while GM₁ and GM₃ did not affect the HA reactions of these strains.

3.3. Other inhibition experiments

The possible role of other glycoconjugates including NANA as possible erythrocyte receptors for CFA/I and K 99 ETEC strains was further investigated. Pre-treatment of sheep and human erythrocytes with clostridial neuraminidase eliminated haemagglutination of the CFA/I strains with human erythrocytes, but the neuraminidase treatment did not affect the haemagglutination reaction of the K 99 strains with the sheep erythrocytes (Table 3).

Pre-treatment of sheep and human erythrocytes with trypsin did not affect HA with either of these two surface antigens, while digestion with Pronase resulted in negative haemagglutination reactions with all the four CFA/I strains. However, the K 99 strains failed to haemagglutinate Pronase-treated sheep erythrocytes.

TABLE 3

Haemagglutination of CFA/I and K 99 positive *Escherichia coli* with enzyme treated erythrocytes

<table>
<thead>
<tr>
<th>Type of antigen/strain a</th>
<th>Erythrocyte species</th>
<th>Control haemagglutination</th>
<th>Treatment with c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neuraminidase (1.0 I.U./ml)</td>
</tr>
<tr>
<td>CFA/I</td>
<td>human</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CFA/II</td>
<td>bovine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K 99</td>
<td>sheep</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2683a b</td>
<td>human</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a These antigens show mannose-resistant haemagglutination of the indicated erythrocyte species.

b This strain was isolated from urinary tract infection. Serologically it lacks K 88, K 99, CFA/I and CFA/IIP antigens.

c + indicates positive haemagglutination and – indicates negative haemagglutination reactions.
4. Discussion

The colonizing factors of ETEC share certain properties: (i) they are pilifimbriae)-like structures (CFA/I and CFA/II) or fibrillae-like structures (K 88 and K 99 antigens) [9]; (II) they are all plasmid-mediated; (iii) they confer haemagglutinating properties and show preferential agglutination of erythrocytes of certain animal species as follows: guinea pig erythrocytes by K 88 antigen, sheep erythrocytes by K 99 antigen, human erythrocytes by CFA/I and bovine erythrocytes by CFA/II; (iv) the haemagglutination reactions caused by these structures are resistant to inhibition by D-mannose [1,3,4,8].

Recent studies indicate that hydrophobic interactions may be important in the binding of piliated E. coli and gonococci to eukaryotic cells [9,15]. However, the strict specificity in attachment of such piliated organisms to certain epithelial cell surfaces may be controlled by more specific interactions such as biospecific lectin-like interactions between the bacterial and animal cell surface [7]. D-Mannose inhibits HA of piliated E. coli (type 1 pilus) [7]. L-fucose, at high concentration inhibits attachment of cholera vibrios to intestinal brush border [4]. These observations are basis for the proposal of bacterial lectin-like antigens showing sugar-specific interaction with glycoconjugates such as glycolipids and glycoproteins on eukaryotic cells and erythrocytes of various species.

Buchan and co-workers [15] reported that different gangliosides at less than 1 μM concentration inhibited attachment of gonococcal pili to human buccal cells. GD1b produced more inhibition than GD1a>GM1>GT. In contrast, these gangliosides did not show any inhibitory effect on the attachment of E. coli.

It was concluded that hydrophobic interactions and decreases in repulsive charged forces were important in this process. Heparin and to a lesser extent other acidic polymers inhibited attachment and highly positively charged polymer-like protamine sulphate-enhanced pilus attachment [15].

Studies on hormones show that both electrostatic and hydrophobic interactions are involved in promoting binding of the hormone molecule (e.g. thyrotropin, TSH) to cell surface receptors [16]. This results in exclusion of water molecules and salt from the interacting regions of the hormone molecule and the exposed hydrophobic regions of the ganglioside receptor or with the lipid parts of the ganglioside micelles or cell membrane [16]. Recent studies on hormone and tetanus toxin interactions with cell receptors indicate that both ganglioside and glycoprotein are involved in a ligand-receptor complex [16]. It is tempting to speculate that both glycolipid(s) and glycoprotein(s) [5] are involved in the binding of CFA/I or K 99 antigen to erythrocyte surface since trypsin treatment did not affect HA by either CFA/I or K 99 antigen, but HA of human erythrocytes by CFA/I antigen was completely eliminated after pronase treatment. HA of K 99 antigen with sheep erythrocytes was not affected after the erythrocytes were treated with pronase. K 99 antigen, on the other hand haemagglutinates human erythrocytes and this reaction seemed to be partially reduced after the human erythrocytes were treated with pronase.

GM1 is resistant to C. perfringens neuraminidase, due to steric hindrance by Gal-NAc moiety [17]. After neuraminidase treatment GM1 is expected to remain intact in erythrocytes that have been treated with this enzyme. This could therefore explain why K 99 haemagglutinates neuraminidase-treated erythrocytes of human and sheep origin. However, the haemagglutination reaction of K 99 antigen with the neuraminidase-treated human erythrocytes is slower than the reaction with the untreated erythrocytes. But this reaction is identical to the reactions with sheep erythrocytes (Table 3). Hence CFA/I and K 99 seem to attach to glycoconjugates resembling GM2, where NANA plays an essential role. In contrast the specificity seems to be less restricted for the moiety in the Gal-NAc position.

Evans and colleagues recently reported inhibition of CFA/I and CFA/II HA of human erythrocytes with NANA and postulated a non-specific charge effect since inhibition was obtained at pH 3 [18]. We were not able to obtain inhibition with NANA after pH adjustment (without pH adjustment erythrocytes lysed). We also conclude that non-specific charge interactions may not be very important in the HA reaction of the various piliated E. coli, since heparin did not inhibit these HA reactions.

The concentration of ganglioside that is necessary to inhibit HA as reported here is in agreement with the reported concentrations of gangliosides used to
study binding of different bacterial toxins with mammalian receptors [16,19]. Cmc values are extremely small. Consequently care should be taken in interpreting the effects of interaction in these studies [20]. However, more conclusive evidence of specific interaction between protein molecules and glycolipids can be obtained in studies performed with amphiphilic substances which have been incorporated into liposomes [21].

Our observations suggest that both glycolipids and glycoprotein may be involved in the erythrocyte receptor for these two bacterial HA antigens (haemagglutinins). Further work is being done now in order to investigate this hypothesis, and future studies will reveal if the nature of the GM2-like erythrocyte receptor is identical or similar to the natural receptor of the intestinal epithelial cell surface.

Acknowledgements

This study was supported by grants from the Swedish Medical Research Council (16X-4723) and the Swedish Agency for Research and Cooperation with Developing Countries. We are grateful to B. Ersson, S. Hjerten, S. Höglund, and H. Wigzell for stimulating and helpful discussions.

References