Binding of porcine transmissible gastroenteritis virus by enterocytes from newborn and weaned piglets

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ABSTRACT

Enterocytes were harvested by chelation in a series of seven fractions from the tips of the villi to the crypts of the jejunum of newborn or weaned piglets. Binding of the low cell culture passaged Miller-6 strain of transmissible gastroenteritis virus (TGEV) to villous enterocytes from newborn piglets was at a high level, similar to that observed to cultured swine testis (ST) cells. Binding of the virus to cryptal enterocytes from newborn piglets or to villous or cryptal enterocytes from weaned piglets was significantly lower. In a competitive virus binding assay with radiolabelled virus, the binding of TGEV to ST cells was found to be saturable, while binding to MDBK cells, in which the virus fails to replicate, was at a lower level and was non-saturable. In the same assay, virus binding to the villous enterocytes from the jejunum of a newborn piglet was saturable, while binding to cryptal enterocytes from a newborn piglet, and to villous and cryptal enterocytes from a weaned piglet, was non-saturable. It was concluded that the high susceptibility of newborn piglets to TGEV infection, and the tropism of the virus for villous enterocytes, may relate to the presence of specific, saturable binding sites on the plasma membrane of villous enterocytes.

INTRODUCTION

Transmissible gastroenteritis virus (TGEV) is a porcine enteric coronavirus (Bohl and Pensaert, 1989) which infects the mature enterocytes of the small intestine, leading to villous atrophy, diarrhoea, malabsorption and death in newborn piglets (Saif and Heckert, 1990). The virus does not infect the cryptal enterocytes, and there is also evidence that the enterocytes which replace those lost from the villi during the infection are also resistant to the virus (Pensaert et al., 1970). The tropism of the virus for mature villous enterocytes has not been explained, and a major objective of the present study

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was to determine whether this might relate to the ability of the virus to bind to these cells.

A further feature of the pathogenesis of TGE is that the severity of the disease is inversely related to the age of the piglet (Moon et al., 1973), and it has been postulated that the high sensitivity of the neonate may relate to the slow replacement rate of enterocytes in newborn piglets (Moon, 1971), to the facilitation of viral replication by deep cytoplasmic tubular invaginations in neonatal enterocytes (Wagner et al., 1973) and by the lack of natural killer cell activity in newborn piglets (Cepica and Derbyshire, 1984). The possibility that there might be a decline in enterocyte virus-binding activity with age has not been investigated.

Specific attachment sites for TGEV have been demonstrated in porcine cell culture (Nguyen et al., 1987), and a 32 to 35 kDa protein was subsequently identified as a receptor molecule on swine testis (ST) cells (Flakus et al., 1990). Recently, Delmas et al. (1992) identified aminopeptidase N as a major receptor for TGEV on both ST cells and porcine enterocytes. Aminopeptidase N is also a receptor for the human coronavirus 229E (Yeager et al., 1992) while mouse hepatitis virus attaches to a glycoprotein of the carcinoembryonic antigen family on intestinal brush border and hepatocyte membranes (Williams et al., 1991). The S glycoprotein of the bovine coronavirus was shown recently to attach to a sialic acid receptor on the surface of red blood cells (Schultze et al., 1991), although this virus may also attach to the same receptor by means of the haemagglutinin-esterase glycoprotein (Vlasak et al., 1988), which is lacking in TGEV.

In this paper we describe the binding of TGEV to susceptible porcine and resistant bovine cell cultures, and to enterocytes contained in a series of fractions collected from the tips of the villi to the crypts of the jejunum of neonatal and weaned piglets. As evidence of specific binding to these cells, the saturability of virus binding was determined in a competitive assay with radiolabelled virus.

MATERIALS AND METHODS

Cells

Stable swine testis (ST) cells (McClurkin and Norman, 1966) and Madin-Darby bovine kidney (MDBK) cells (American Type Culture Collection) were cultivated by standard methods.

Enterocytes were harvested as described by Weiser (1973) from the jejunum of eight newborn piglets at 2 or 3 days of age and from four 3 weeks old weaned piglets which were killed with an intravenous overdose of sodium pentobarbitone. An approximately 1 m length of jejunum was rinsed five times with 0.154 M NaCl, 1 mM dithiothreitrol (DTT) and then closed at one end with surgical thread. The intestine was filled with citrate buffer (0.5 mM DTT,

1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, pH 7.3), closed with a clip and incubated in Hanks' balanced salt solution (HBSS) containing 0.5 mM DTT for 15 min at 37°C. The contents of the intestine were then discarded and replaced with magnesium and calcium-free phosphate-buffered saline (PBS) containing 1.5 mM ethylenediamine tetraacetic acid (EDTA) and 0.5 mM DTT. The intestine was incubated for 10 min at 37°C, when the released cells were collected by centrifugation at 1200 g for 5 min and washed three times with PBS. The 10 min cycles of chelation with EDTA were repeated six times to obtain seven fractions of enterocytes. Samples of intestine were fixed and sections were stained with haematoxylin and eosin after each chelation cycle to monitor the progressive detachment of enterocytes.

Alkaline phosphatase activity was determined in each enterocyte fraction as described by Weiser (1973). A volume of 0.1 ml of packed cells was incubated at 37°C for 15 min in 0.5 M Tris-HCl, pH 9.4, 0.3 mM $ZnCl_2$, 10 mM $HgCl_2$ and 0.23 mM p-nitrophenyl phosphate. After incubation, 0.5 ml of 0.5 N NaOH was added to the assay system, and the released p-nitrophenol was determined spectrophotometrically at 420 nm and related to the protein content of the sample which was determined by the Bradford (1976) method with the Bio-Rad protein assay kit II (Bio-Rad, Richmond, California). Periodic acid-Schiff staining of the cells was performed as described by Gratecos et al. (1978).

Virus

The low cell culture passaged Miller-6 strain of TGEV (Welch and Saif, 1988), kindly supplied by Dr. L.J. Saif, Ohio Agricultural Research Development Center, Wooster, Ohio) was cultivated in ST cells maintained in serum-free medium. Plaque assays were also performed on ST cells. For the preparation of radiolabelled virus, TGEV was cultivated in ST cells in the presence of 200 μCi ml^{-1} of ^{35}S-methionine (Amersham, Oakville, Canada). The cell culture supernatant was clarified by centrifugation at 10000×g for 10 min at 4°C, and the labelled virus was pelleted through a 20% sucrose cushion in TES buffer (0.01 M Tris-HCl, 1 mM EDTA, 0.1 M NaCl, pH 7.4) supplemented with 0.05% Tween 80, by centrifugation at 100000×g for 3 h at 4°C.

Virus binding assay

Enterocyte fractions I to VII, ST cells or MDBK cells were incubated at a concentration of 10^6 cells ml^{-1} for 60 min at 37°C with 10^2, 10^3 or 10^4 PFU ml^{-1} TGEV. The cells were removed by centrifugation at 10000×g for 5 min, and the supernatants tested for residual activity by plaque assay in ST cells. The % virus binding was calculated as

$$\left(\frac{\text{PFU Virus added} - \text{PFU residual virus}}{\text{PFU virus added}} \right) \times 100.$$

Each sample of cells was tested in triplicate against each concentration of virus, and the mean % binding was calculated for each sample.

Competitive virus binding assay

The procedure was modified from that described by Schlegel et al. (1982). Enterocytes harvested from the tips of the villi (fractions I and II) or from the crypts (fraction VII) of the jejunum of one newborn and one weaned piglet, ST cells or MDBK cells, at a concentration of 10^4 cells ml^{-1} were incubated with 10^6 PFU ml^{-1} of TGEV, or with Eagle's minimum essential medium (EMEM), for 4 h at $37°C$. To each sample, ^{35}S-methionine labelled TGEV was added, in concentrations ranging from 10^{-2} to 10^3 PFU/cell, and incubation was continued for 45 min at $37°C$. The cells in each sample were then pelleted by centrifugation at $10000 \times g$ for 5 min, and the pellets resuspended in 100 μl of 1% Triton X-100 (Sigma Chemical Co., St. Louis, Missouri) in PBS. A portion (75 μl) of the suspension was transferred into a scintillation vial with 10 ml of Universal scintillation cocktail (ICN Biomedicals, Irvine, California) and the radioactivity measured as counts min^{-1} (cpm) in a liquid scintillation counter. The assay was run in triplicate on each sample, and mean values obtained. The cpm obtained from the cells preincubated with EMEM represented total virus binding, while the counts obtained after preincubation with 10^6 PFU ml^{-1} of unlabelled TGEV represented non-saturable virus binding. Saturable binding was obtained by subtracting the non-saturable binding cpm from the total binding cpm.

RESULTS

Virus binding to ST and MDBK cells

Virus binding to MDBK cells was less than 15% for each of the three concentrations of TGEV, while for ST cells the mean $\pm$ SD % virus binding was 39 ± 22, 48 ± 27 and 70 ± 5 for concentrations of TGEV of 10^2, 10^3 and 10^4 PFU ml^{-1} respectively. In the competitive virus binding assays, there was no evidence that the binding of TGEV to MDBK cells was saturable, while for ST cells a typical hyperbolic saturable binding curve was obtained (Fig. 1), indicating the presence of saturable binding sites for TGEV on these cells.

Virus binding to enterocytes

Histological examination of sections of intestine after each fraction was harvested revealed that the enterocytes were released progressively from the tips of the villi (fraction I) to the crypts (fraction VII) as chelation with EDTA proceeded. Representative sections are shown in Fig. 2. No crypt cells
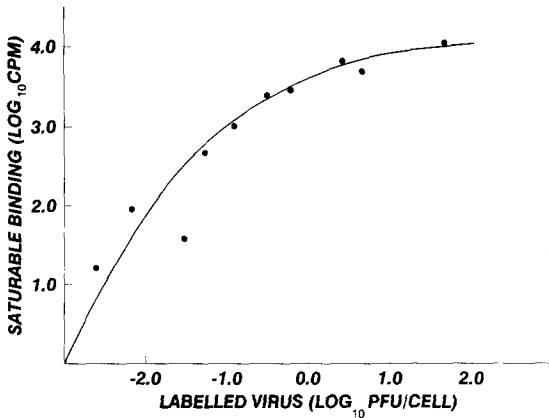
Fig. 1. Saturable binding of radiolabelled TGEV to ST cells. Increasing amounts of labelled TGEV were incubated with ST cells and virus binding was determined as cpm for cells preincubated in EMEM (total binding) or in unlabelled virus (non-saturable binding). Saturable binding was calculated by subtraction of non-saturable binding cpm from total binding cpm.

remained in the jejunum of the newborn piglets after the harvesting of fraction VII, while in the weaned piglets, small numbers of cells remained deep in the crypts after the removal of this fraction. These could be released by chelation for a further 20 min. The highest levels of alkaline phosphatase activity (mean $1330 \pm SD\ 400$ mU mg^{-1} protein) were found in the enterocytes contained in fraction I from four piglets killed at 2 days of age. The activity in the same fraction from the weaned piglets was 360 ± 93 mU mg^{-1} protein. The remaining fractions, from both newborn and weaned piglets had lower levels of activity than fraction I, ranging from 290 ± 150 mU to 500 ± 280 mU in the newborn piglets and from 114 ± 64 mU to 152 ± 59 mU mg^{-1} protein in the weaned piglets, and there were no consistent differences in activity among fractions II to VII. The periodic acid-Schiff stained preparations of enterocytes showed a progressive reduction in staining intensity from fractions I to VII in both the newborn and weaned piglets.

In the virus binding assays, similar results were obtained for each of the three concentrations of virus which were used, and the findings when 10^4 PFU ml^{-1} of TGEV were added to the enterocyte fractions are shown in Fig. 3. High levels of virus binding were obtained with the enterocytes in fractions I and II, derived from the tips of the villi of newborn piglets, with relatively low binding to enterocytes in the remaining fractions from the newborn piglets, and in all the enterocyte fractions from the weaned piglets. When analysed by the Student's t-test, binding was significantly higher ($P < 0.05$) to enterocytes in fractions I and II from the newborn piglets than in all other enterocyte fractions, among which significant differences in virus binding did not occur.

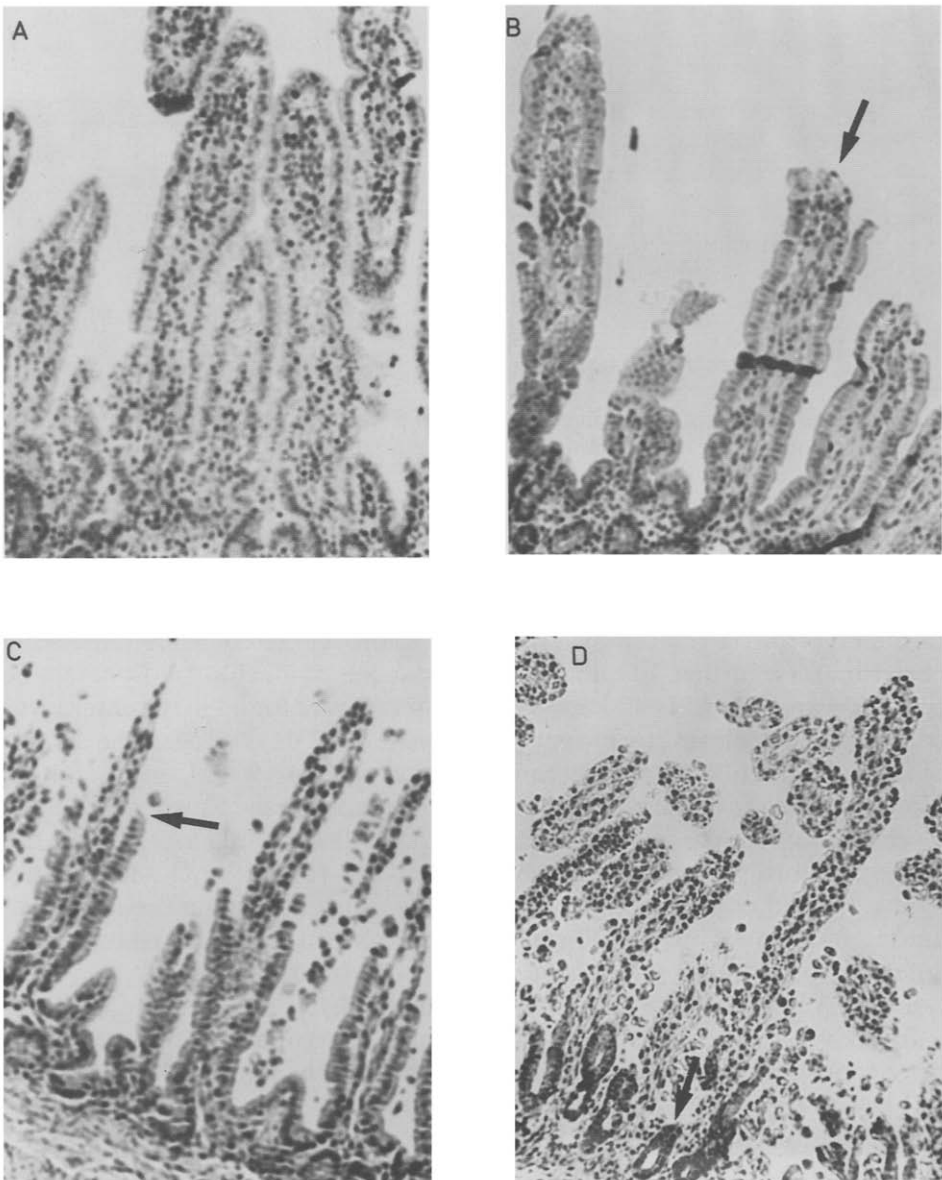In the competitive virus binding assays, ST and MDBK cells were included

Fig. 2. Histological sections, stained with haematoxylin and eosin, of jejunum of a newborn piglet, A: – before chelation with EDTA; B: – after one cycle of chelation – enterocytes have been released from the tips of the villi (arrow); C: – after three cycles of chelation – enterocytes have been released from approximately one half of each villus (arrow); D: – after six cycles of chelation – enterocytes have been released completely from each villus, but some cryptal enterocytes remain (arrow).
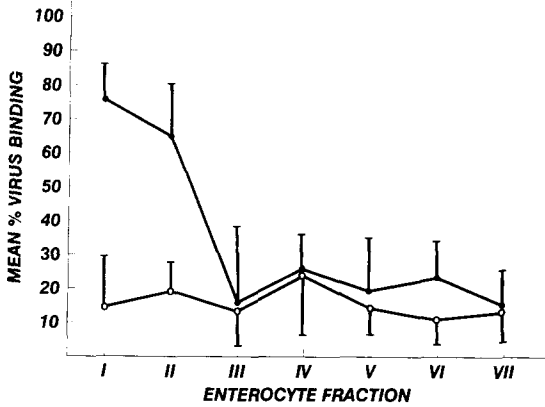
Fig. 3. Mean % virus binding of 10^4 PFU ml^{-1} of TGEV to enterocytes (10^6 cells ml^{-1}) in seven fractions from the villi (fraction I) to the crypts (fraction VII) of the jejunum from eight newborn piglets (●) and from four weaned piglets (○). Vertical bars represent standard deviations. Virus binding to fractions I and II from the newborn piglets was significantly higher than to all other fractions.
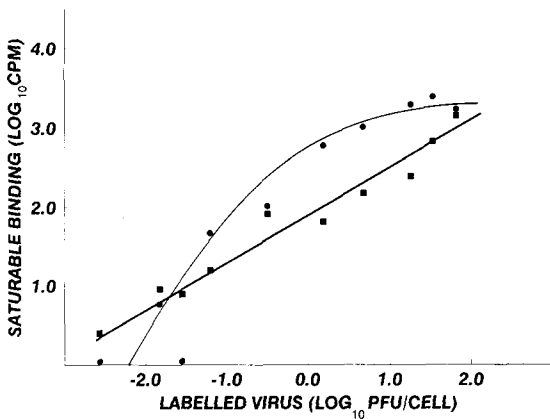

Fig. 4. Saturable binding of radiolabelled TGEV to enterocytes harvested from the tips of the villi (●) or the crypts (■) of the jejunum from a newborn piglet. Increasing amounts of labelled TGEV were incubated with enterocytes and virus binding was determined as cpm for cells preincubated in EMEM (total binding) or in unlabelled virus (non-saturable binding). Saturable binding was calculated by subtraction of non-saturable binding cpm from total binding cpm.

in each assay as positive and negative controls for saturable virus binding, and the results obtained confirmed the findings reported above for these cells. Of the enterocyte fractions harvested from the tips of the villi or the crypts of a newborn or a weaned piglet, and tested in the competitive virus binding assay, only the enterocytes harvested from the villi of the newborn piglet showed saturable binding of TGEV (Fig. 4), similar to that demonstrated for

ST cells. Binding of TGEV to the other three fractions, like the binding of the virus to MDBK cells, was non-saturable. Binding of TGEV to enterocytes from the crypts of the newborn piglet is shown in Fig. 4, and similar results were obtained with enterocytes from the villi and crypts of the weaned piglet.

DISCUSSION

Studies on the attachment of TGEV to ST cells were first described by Nguyen et al. (1987), who concluded that the attachment was to specific sites on the cell surface since incubation of the cells with one strain of TGEV inhibited the attachment of another strain of the virus. The present findings on the saturability of virus binding to ST cells support this conclusion. Binding of TGEV to MDBK cells, in which the virus fails to replicate, was at a much lower level than to ST cells, and since the binding to MDBK cells was non-saturable, it is concluded that these cells lack specific receptors for TGEV.

The technique of release of enterocytes, by chelation, from the tips of the villi to the jejunal crypts, developed initially for use in rats (Weiser, 1973) was successfully applied to piglets. Histological examination of the intestine after each cycle of chelation confirmed the progressive release of enterocytes in each fraction. A high level of alkaline phosphatase activity was found only in the enterocytes harvested from the tips of the villi. This contrasted with findings in the rat (Weiser, 1973) in which there was a progressive decline in enzyme activity from the villi to the crypts. However, in the present study there was a progressive decline in the intensity of periodic acid-Schiff staining, from the villi to the crypts, as described in the rat (Gratecos et al., 1978), corresponding to the glycoprotein content of the plasma membrane, which increases with progressive differentiation of the enterocytes.

Binding of TGEV to the villous enterocytes of newborn piglets occurred at a level comparable to that demonstrated for ST cells, and significantly higher than virus binding to all other enterocyte fractions from both newborn and weaned piglets. The competitive binding assays indicated that saturable binding occurred only to the villous enterocytes of newborn piglets. Since saturable binding is accepted as evidence of binding to specific receptors on the cell surface (Tardieu et al., 1982) this finding would suggest that specific TGEV receptors on enterocytes are restricted to neonatal villous enterocytes. This could be an important factor contributing to the susceptibility of villous, rather than cryptal enterocytes, to infection with TGEV. The apparent lack of specific virus binding to villous enterocytes from weaned piglets was a somewhat puzzling finding since the virus is capable of infecting these cells in older piglets, although the latter are much less susceptible to infection than newborn piglets. Our use of a cell culture adapted strain of TGEV which is somewhat less virulent than some field strains of the virus may have contributed to this result, but it may be that specific receptors are far fewer in older piglets, and

below the level of detection by the methods used in the present study. A low level of specific binding may have been masked by non-specific binding of the virus to the cell surface. It is also possible that the non-specific binding of TGEV to villous enterocytes may lead to productive infection, but much less efficiently than binding to specific receptors. It has been shown that the dose of virus needed to infect an adult pig is 10^4 times greater than that required to infect a neonate (Witte and Walther, 1976) and this may reflect the sparseness of receptors on the villous enterocytes of older pigs.

It is concluded that the high susceptibility of newborn piglets to TGEV, and the tropism of the virus, may be related to the presence of specific, saturable binding sites on the plasma membrane of the villous enterocytes in the neonate. Shepherd et al. (1979) observed TGE viral particles traversing the intact microvilli of mature enterocytes in the small intestine of an experimentally infected piglet, and postulated the synthesis of brush border receptors as the enterocytes differentiated during their migration from the crypts to the tips of the villi. The present findings provide the first evidence in support of such a hypothesis. The relationship of the aminopeptidase N receptor for TGEV, demonstrated recently by Delmas et al. (1992), to our findings, remains to be explored by determining the distribution of this enzyme in different enterocyte fractions from newborn and weaned piglets.

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