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Evidence for a Putative Second Receptor for Porcine Transmissible Gastroenteritis Virus on the Villous Enterocytes of Newborn Pigs

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Aminopeptidase-N (APN) has been identified [B. Delmas, J. Gelfi, R. L'Haridon, L. K. Vogel, H. Sjostrom, O. Noren, and H. Laude, Nature (London) 357:417–420, 1992] as a major receptor for porcine transmissible gastroenteritis virus (TGEV). Binding of TGEV to villous enterocytes from the jejuna of newborn pigs is saturable and at a higher level than that of binding of virus to newborn cryptal enterocytes or to enterocytes from older piglets (H. M. Weingartl and J. B. Derbyshire, Vet. Microbiol. 35:23–32, 1993). The distribution of APN in enterocytes in the jejuna of neonatal and 3 week-old-piglets, as determined by the measurement of enzymatic activity and by labeling of the cells with an anti-APN monoclonal antibody, did not correspond with the reported distribution of saturable binding sites on enterocytes. Monoclonal antibodies, which were prepared against plasma membranes derived from enterocytes harvested from the upper villi of newborn pigs, blocked the replication of TGEV, but not the porcine respiratory coronavirus, in ST cells and immunoprecipitated a 200-kDa protein in ST cell lysates. This protein was demonstrated by immunohistochemistry and by fluorescence-activated cell scanning to be present on the villous enterocytes of newborn pigs but to be lacking on the cryptal enterocytes of newborn pigs and on the villous and cryptal enterocytes of 3-week-old piglets. Since this distribution of the protein corresponds to the previously demonstrated distribution of saturable binding sites, we conclude that the 200-kDa protein may be an additional receptor for TGEV which is restricted to the villous enterocytes of newborn pigs and which contributes to the age sensitivity of these animals to the virus.

Transmissible gastroenteritis virus (TGEV) is an enteric coronavirus which causes severe and frequently fatal diarrhea in newborn pigs, while the clinical signs in older piglets or in adult pigs are mild or inapparent (14, 15). It has also been shown experimentally that much higher doses of virus are required to produce disease in adult pigs than in newborn pigs (26). Histopathological studies of TGEV-infected piglets have shown that the absorptive epithelial cells on the villi of the small intestine are the main targets for the virus, which was not detected in the cryptal enterocytes or in the villous cores (12, 13).

It has been suggested that several factors may contribute to the severity of TGE in newborn pigs. These include the relatively low replacement rate of enterocytes in newborn pigs (9); the presence of enterocytes of fetal origin, containing a prominent tubulo-vacuolar system which might facilitate virus replication, on the villi of newborn pigs (22); and the lack of natural killer activity in the intraepithelial lymphocytes of newborn pigs (2). The preferential tropism of the virus for the villous enterocytes of newborn pigs could be related to the presence of specific high-level virus-binding sites on these enterocytes, which are lacking on the cryptal enterocytes and on the enterocytes of older piglets (23).

Aminopeptidase-N (APN) has been identified as a major receptor for TGEV on both swine testis (ST) cells and porcine enterocytes (3). APN has also been identified as a receptor for the human coronavirus 229E (28). Since APN belongs to the prominent group of intestinal microvillar peptidases involved in the final hydrolysis of ingested nutrients (11), its presence could explain the intestinal tropism of TGEV, but not the preferential tropism of the virus for the villous enterocytes of newborn pigs. In the present studies, we confirmed that the distribution of APN in enterocytes from the villi and crypts of newborn and older piglets, as determined by the measurement of enzymatic activity and by labeling of the cells with an anti-APN monoclonal antibody (MAb), did not correspond with the previously determined distribution of high-level binding sites for the virus (23). By means of a MAb prepared against plasma membranes derived from villous enterocytes harvested from newborn pigs, we have identified a 200-kDa protein in ST cells and in villous enterocytes in newborn pigs which may be a second receptor for TGEV and which may contribute to the age sensitivity of these animals to the virus.

MATERIALS AND METHODS

Cells. The ST and MDBK cell lines and NS-1 myeloma cells were obtained from the American Type Culture Collection (ATCC) and were cultivated by standard methods. Porcine enterocytes were collected from the jejuna of nursing 3-day-old piglets and from 3-week-old weaned piglets. The piglets were obtained from a specific-pathogen-free herd of Yorkshire swine which were serologically negative for antibodies against TGEV and the porcine respiratory coronavirus (PRCV). They were killed by an intravenous overdose of sodium pentobarbitone, and the enterocytes were harvested in seven fractions from the tips of the villi to the crypts of the jejunum by chelation with EDTA as described previously (23). It was determined histologically that the enterocytes in fractions I and II were derived from the upper third of the villus and that those in fractions III and IV were from the middle third of the villus, while the enterocytes in fractions V and VI were from the lower third of the villus and those in fraction VII were from the crypt (23). Alkaline phosphatase activity was determined as described elsewhere (24), and protein content was measured

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by the method described by Bradford (1). APN activity (20) in 50 mM Tris-HCl buffer (pH 7.3) containing 1 mM L-leucine-4-nitroanilide was determined at 37°C. Release of 4-nitroaniline was measured spectrophotometrically at 410 nm, with porcine microsomal kidney APN (Sigma) as a standard.

Viruses. The attenuated, cell culture-adapted Purdue strain (ATCC) and the low-cell-culture-passaged Miller-6 strain (25) of TGEV were kindly provided by L. J. Saif, Ohio Agricultural Research and Development Center, Wooster, Ohio. The Ambico and Diamond strains of the virus were isolated from commercial vaccines. The PRCV was kindly provided by H. Laude, Institut National de la Recherche Agronomique (INRA), Jouy-en-Josas, France. The viruses were cultivated and assayed in ST cells by standard methods.

Preparation of plasma membranes. Plasma membranes were prepared from enterocytes harvested from the upper villi of the small intestine of newborn pigs by a modification of a previously described method (4). The harvested cells were washed with phosphate-buffered saline (PBS), pelleted, and resuspended in a buffer consisting of 10 mM Tris-HCl, 5 mM $MgCl_2$, 0.15 M sucrose, 5 mM NaH_2PO_4, 30 mM succinic acid, and 1 mM $MnCl_2$ (pH 7.4). The cells were disrupted by sonication, and the suspension was stirred for 30 min at room temperature. The suspension was then centrifuged at 4°C for 5 min at 15,000 × *g*, and the supernatant was collected and centrifuged again at 4°C for 60 min at 105,000 × *g*. The pellet was resuspended in PBS and stored at −70°C. Ouabain-sensitive ATPase (17) was monitored at each stage, and enrichment of 1.6 to 1.8 times was obtained in the partially purified preparations compared with the crude plasma membranes, a result which was comparable with previously published data (4).

MAbs. The G43 anti-APN MAb (3) was kindly provided as ascitic fluid by B. Delmas, INRA; an irrelevant MAb, which was directed against a surface protein of *Rhodococcus equi*, was a gift from J. F. Prescott, Department of Veterinary Microbiology and Immunology, University of Guelph; and a second irrelevant MAb, which was directed against ovine tau interferon (IFN-τ), was donated by R. L'Haridon, INRA. MAbs were prepared against partially purified plasma membranes from the upper villous enterocytes of newborn pigs. BALB/c mice were given one subcutaneous injection and then four intraperitoneal booster inoculations, and the splenocytes were fused with NS-1 myeloma cells using polyethylene glycol (Sigma). Hybridomas were selected in hypoxanthine-aminopterin-thymidine (HAT; Sigma) medium and were screened for antibody production in an enzyme-linked immunosorbent assay (ELISA) in which the plates were coated with a sodium dodecyl sulfate (SDS) lysate of ST cells and blocked with 3% skim milk powder in PBS-Tween 20 (0.05% [vol/vol]) for 30 min at 37°C. The hybridoma supernatants were incubated on the plates for 60 min at 37°C, and bound antibodies were detected by incubation for 60 min at 37°C with horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) or IgM antibodies (Bio-Rad). The hybridoma supernatants which were positive in this ELISA were further screened for their abilities to block binding of TGEV to ST cells in a cytopathic effect reduction assay (CPERA). The classes of antibodies in hybridoma supernatants which tested positive in the CPERA were determined by an ELISA with a goat anti-mouse antibody kit obtained from Southern Biotechnology Associates, Inc. The MAbs which blocked virus binding in the CPERA (MAbs 55, 166, 244, and 318), together with the G43 MAb, were tested for anti-APN activity in ELISAs in which the plates were coated with porcine kidney cytosol APN or porcine kidney microsomal APN (Sigma).

CPE reduction assays. For the screening of the hybridoma supernatants described above, ST cells were grown to confluency in 96-well flat-bottom plates (Nunclon) and treated with 100 μl of hybridoma supernatant per well for 60 min at room temperature and then for 10 min at 37°C. The wells were then inoculated with 50 μl of TGEV containing 10^6 PFU per ml and were incubated for 35, 50, or 80 min at room temperature followed by 10 min at 37°C. The supernatants and virus were then aspirated and replaced with 200 μl of hybridoma supernatant. The plates were then incubated at 37°C until the virus control wells, without hybridoma supernatant, showed complete CPE, at which point the protection of the cells against virus challenge by the antibodies was evaluated. The CPERA was also performed using PRCV and the G43 and 166 MAbs, with the irrelevant anti-IFN MAb as a negative control. In this test, the G43 ascitic fluid was diluted 1:100 or 1:1,000, and the 166 and anti-IFN hybridoma supernatants were used undiluted. After treatment as above with the appropriate antibodies, the ST cell monolayers were challenged with 50 μl of the Purdue strain of TGEV or PRCV, each containing 10^5 median cell culture infectious doses per ml. The test was evaluated when full CPE appeared in the PRCV controls. The CPERA was also used to determine the combined effect of G43 and 166 MAbs in blocking the CPE of TGEV in ST cells. Twofold dilutions of MAb G43 from 1:400 to 1:51,200 were prepared in Eagle's minimum essential medium, in the irrelevant anti-IFN MAb, or in undiluted MAb 166 or MAb 166 diluted 1:2. After treatment with the antibodies as described above, the ST cells were challenged with 6×10^6 PFU of the Purdue strain TGEV per ml and were incubated until CPE appeared in the control cells that had been treated with the 166 MAb alone and challenged with TGEV.

Immunoprecipitation of ST cell lysates. ST cells were grown to 80 to 100% confluency in 6-well plates (Nunclon) and labeled with 2 ml of 100 μCi of [^{35}S]methionine (ICN Biochemicals) per ml for 6 h. The cells were then washed twice with cold PBS and lysed with 200 μl of lysis buffer (20 mM *N*-2-hydroxy ethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.8], 150 mM NaCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 2% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride). The lysates were stored at −70°C. For immunoprecipitation, 200-μl volumes of radiolabeled ST cell lysate were homogenized, centrifuged at 16,000 × *g* for 30 min at 4°C, and precleared with 15 μl of recombinant protein G (rPG) beads (Gibco) for 60 min at room temperature. Then, 200 μl of MAb, in the form of undiluted hybridoma supernatant or G43 ascitic fluid diluted 1:100 in PBS, was added to the cell lysate, and the lysate was incubated for 60 min at room temperature. The samples were then centrifuged at 10,000 × *g* for 10 min, and 20 μl of rPG was added to the supernatant, which was incubated for 60 min at room temperature. The rPG agarose beads with bound antigen-antibody complexes were pelleted by centrifugation at 16,000 × *g* for 2 min, washed with buffers A (1 M NaCl, 0.01 M Tris-HCl [pH 7.2], 0.1% [vol/vol] Nonidet P-40), B (0.1 M NaCl, 1 mM EDTA, 0.01 M Tris-HCl [pH 7.2], 0.1% [vol/vol] Nonidet P-40, 0.3% SDS), and C (0.01 M Tris-HCl [pH 7.2], 0.1% [vol/vol] Nonidet P-40), and resuspended in 35 μl of polyacrylamide gel electrophoresis (PAGE) sample buffer. Precipitation with the MAbs of the IgM class (MAbs 244 and 318) was attempted with rPG or goat anti-mouse IgM (μ-chain specific)-coated agarose beads (Sigma). MAb G43 was used as a positive control, and rPG or anti-IgM agarose beads were used as negative controls. Labeled MDBK cell lysate was used in place of the ST cell lysate as a negative control.

SDS-PAGE and Western immunoblotting. SDS-PAGE was

performed on the immunoprecipitated ST cell proteins by a minigel modification of a method described elsewhere (8), using 8% resolving gel and 100 V. The gels were either stained with Coomassie brilliant blue, treated with enhancer (En^{3}Hance; DuPont), dried, and used for autoradiography, or were used for protein transfer after equilibration in transfer buffer (25 mM Tris, 192 mM glycine [pH 8.3], 20% [vol/vol] methanol). The resolved proteins were transferred onto polyvinylidene difluoride membranes (Immobilon P; Millipore) in a wet miniblot apparatus (Bio-Rad) at 30 V for 18 h at 4°C. Immunoprobing was then performed according to the Gibco Immunoselect instruction manual, using goat anti-mouse IgG horseradish peroxidase-labeled enzyme immunoassay grade antibodies (Bio-Rad). Endoglycosidase H treatment of the immunoprecipitated proteins resolved by SDS-PAGE was performed according to the instructions supplied with the enzyme (Boehringer Mannheim Biochemicals). Deglycosylation of the antibodies used in the immunoprecipitation was used as a control for the activity of the enzyme.

Competitive ELISA between TGEV and MAbs. The competitive ELISA procedure was based on the ELISA which was used to screen the hybridoma supernatants for reactivity with ST cell lysates, except that before incubation with the MAbs, the coated plates were incubated for 1 or 2 h at 37°C with 50 μl of TGEV containing 10^5 PFU/ml. The MAbs were diluted according to a calibration curve obtained in the screening ELISA so that the lowest dilution of an antibody still giving the highest mean absorbance reading (0.462 for MAb G43 and 0.250 for MAb 166) was used in the competitive ELISA. The blocking of antibody binding by the virus was calculated from the difference in mean absorbances between the wells with MAb only (A) and wells preincubated with virus (B), so that the percentage of blocking was $[(A - B)/A] \times 100$.

Immunohistochemistry. ST cells, MDBK cells, and sections of the jejuna of newborn and weaned piglets were immunostained with the streptavidin-biotin system for immunological staining (Zymed Laboratories). The cultured cells were fixed in 80% acetone, and the tissue sections were fixed in 10% paraformaldehyde. Endogenous peroxidase was blocked by treatment with 3% H_2O_2 in methanol for 30 min at room temperature. Some sections were treated with 0.25% trypsin for 8 min at 37°C. Nonspecific antibody attachment sites were blocked with 10% normal rabbit serum for 20 min at 37°C. The slides were then incubated with MAb for 60 min at 37°C, and after incubation with the biotinylated secondary antibody for 10 min at room temperature, the slides were flooded with enzyme conjugate and then by the substrate chromogen mixture. Hematoxylin was used as a counterstain.

FACS. ST cells and harvested porcine enterocytes were washed three times in PBS and dispensed in 100-μl volumes containing 10^6 cells per ml into 96-well round-bottom microtiter plates (Nunclon). The plates were centrifuged for 5 min at low speed (1,000 rpm; Beckman TJ-6), and the supernatant was replaced with 100 μl of the primary MAb. After incubation for 45 min at 4°C, the cells were washed twice with 0.1% sodium azide in PBS, and 25 μl of biotinylated rabbit anti-mouse secondary antibody (Zymed Laboratories) was added to each well. The plates were then incubated for a further 45 min at 4°C, after which the antibody was removed and the plates were washed twice as described above. Finally, 25 μl of streptavidin-phycoerythrin (Becton Dickinson) was added to each well, and the wells were incubated for 15 min at 4°C. The washed cells were then resuspended in 0.5% paraformaldehyde in PBS, and the suspensions were stored overnight at 4°C before scanning on a fluorescence activated-cell scanner (FACS; Becton Dickinson).

TABLE 1. Distribution of alkaline phosphatase and APN activities in porcine enterocyte fractions

Enterocyte fraction[a]	Alkaline phosphatase (mU/mg of protein)				APN (mkat/kg of protein)			
	Newborn pig		3-wk-old piglet		Newborn pig		3-wk-old piglet	
	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
I	964	1,000	630	710	3.1	2.5	2.2	2.2
II	632	639	740	740	2.7	2.2	2.0	2.4
III	519	439	470	520	2.7	1.6	1.9	2.1
IV	421	338	330	430	2.9	1.6	1.7	3.0
V	464	285	250	266	2.6	2.0	1.6	2.1
VI	450	312	235	623	1.8	1.7	1.6	2.3
VII	357	284	110	245	2.2	2.1	1.9	1.1

[a] Fractions I and II were derived from the upper third of the villus, fractions III and IV were derived from the middle third, fractions V and VI were derived from the lower third, and fraction VII contained cryptal enterocytes.

RESULTS

Distribution of APN enzymatic activity in porcine enterocyte fractions. To determine whether the distribution of APN corresponded with the previously described high levels of saturable binding of TGEV (23), enterocytes were harvested from two newborn and two 3-week-old piglets as a series of seven fractions from the tips of the villi to the crypts and were assayed for APN enzymatic activity. The same fractions were also assayed for alkaline phosphatase activity, which has been shown to decline progressively from the villi to the crypts (24). Similar levels of APN activity were found in newborn and older piglets (Table 1), and there were no consistent differences in the levels of activity among the various fractions. In contrast, levels of alkaline phosphatase activity in the crypts that were lower than those in the villi were found.

Preparation of MAbs. In an attempt to demonstrate an additional receptor for TGEV on the villous enterocytes of newborn pigs, MAbs were prepared from plasma membranes harvested from these cells. Supernatants from 18 of the more than 600 hybridomas which were produced reacted in the screening ELISA with the ST cell lysate, and these 18 clones were further tested for blocking of Miller-6 TGEV replication in ST cells by the CPERA. Four of these hybridomas (nos. 55, 166, 244, and 318) produced antibodies which blocked the replication of the virus. MAbs 244 and 318 were classified as IgM, MAb 166 was classified as IgG3, and MAb 55 was classified as IgG1. Blocking of virus replication was also obtained when the CPERA was repeated with MAb 166 against the Purdue-, Diamond-, and Ambico cell culture-adapted strains of TGEV. PRCV replication in ST cells in the CPERA was blocked by treatment of the cells with both dilutions of MAb G43, but not by MAb 166. When MAbs 55, 166, 244, and 318 (together with antibody G43 as a positive control) were reacted in an ELISA against porcine kidney cytosol APN or porcine kidney microsomal APN, positive reactions were obtained only with G43 and 244 antibodies.

Immunoprecipitation of ST cell lysates. To identify the specificity of the MAbs, they were used in the immunoprecipitation of radiolabeled ST cell lysate. Autoradiographs of the proteins precipitated by antibodies 244 and 318, with anti-mouse IgM agarose beads and resolved by SDS-PAGE revealed a large number of protein bands, while the autoradiographs of the proteins precipitated by MAb 166 or MAb 55 with rPG indicated that a 200-kDa protein was precipitated

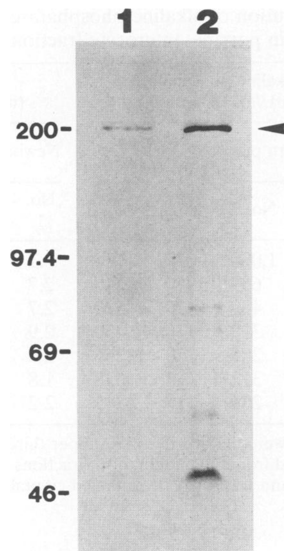

FIG. 1. Autoradiograph of ST cell proteins immunoprecipitated with MAb 55 (lane 1) or MAb 166 (lane 2). ST cells were metabolically radiolabeled with [^{35}S]methionine, and the lysate was immunoprecipitated with MAb. The MAb-protein precipitates were removed from the lysate by rPG on agarose beads and were separated by SDS-PAGE. A protein band in the molecular mass range of 200 kDa (arrowhead) was specifically immunoprecipitated by MAbs 55 and 166. The 50-kDa protein seen in lane 2 was also precipitated in negative controls by rPG in the absence of primary antibody. The positions of molecular mass markers are shown to the left of lane 1.

from the ST cell lysate (Fig. 1). The 50-kDa protein seen in lane 2 of Fig. 1 was also precipitated in negative controls by rPG, in the absence of primary antibody. The specificity of immunoprecipitation was confirmed by Western blotting of immunoprecipitates transferred onto polyvinylidene difluoride membranes after SDS-PAGE. The blots were probed with MAb 166 (Fig. 2). The antibody recognized the 200-kDa

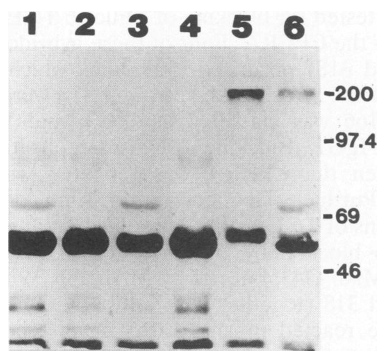

FIG. 2. Western blot of immunoprecipitated ST cell proteins transferred to an Immobilon membrane. ST cell proteins were immunoprecipitated with MAbs 244 (lane 1), 318 (lane 3), 166 (lane 5), and 55 (lane 6) and were probed with MAb 166. The 200-kDa protein was specifically recognized only in the MAb 55 and MAb 166 precipitates. The bands in the 25- to 50-kDa range are ascribed to the primary antibodies in the immunoprecipitates. The primary antibody was detected with horseradish peroxidase-labeled secondary antibody. Lanes 2 and 4 are antibody controls. The positions of molecular weight markers are shown to the right of lane 6.

protein only in the blots derived from immunoprecipitation with antibodies 55 and 166 and failed to recognize any protein immunoprecipitated by antibodies 244 and 318. The bands in the 23- to 50-kDa range were considered to arise from reaction of the primary and secondary antibodies. The molecular mass of the 200-kDa protein was not changed by endoglycosidase H treatment, and the anti-APN MAb G43 precipitated a protein with a molecular mass of 150 kDa from the ST cell lysate (data not shown).

Competitive ELISA between TGEV and MAbs. The competitive ELISA between TGEV and MAbs was used to determine whether MAb 166 or G43 would compete with TGEV for binding to ST cell lysate. MAb 310 was used as a negative control. This antibody reacted with ST cell lysate in the screening ELISA but failed to block binding of TGEV to ST cells in the CPERA. The Miller-6 strain of TGEV blocked 30% of the binding of MAb 166 after incubation for 1 or 2 h, 24% of the binding of antibody G43 after incubation for 2 h, and 15% after 1 h, while only 5.5% of the binding of MAb 310 was blocked by prior incubation of the ST cell lysate with TGEV.

Blocking of TGEV binding by a combination of MAbs G43 and 166. An experiment to determine whether MAbs G43 and 166 might act additively to block the binding of TGEV to ST cells in the CPERA was performed. When the ST cells were treated with MAb G43 in Eagle's minimum essential medium or in the irrelevant antibody, they were protected against CPE up to a dilution of G43 of 1:3,200; however, when MAb G43 was diluted in MAb 166 or in a 1:2 dilution of MAb 166, CPE was blocked in the ST cells up to dilutions of MAb G43 of 1:51,200 or 1:25,600 respectively. Thus, mixtures of MAbs G43 and 166 seemed to have an additive protective effect on ST cells against challenge with TGEV.

Determination of distribution of APN and 200-kDa protein on porcine enterocytes by immunohistochemistry. The immunostaining reaction was first standardized on cell cultures. When either MAb G43 or 166 was used as the primary antibody on ST cells, virtually all of the cells were stained, while no staining of MDBK cells was observed with the same antibodies. No staining of either ST or MDBK cells was obtained when the irrelevant anti-*R. equi* MAb was used as the primary antibody. Sections were then prepared from the jejuna of two newborn and two 3-week-old piglets and were treated with the same reagents. When MAb 166 was used as the primary antibody, specific staining of groups of enterocytes located on the upper villi of newborn pigs was obtained (Fig. 3A). There was no specific staining with this antibody of cryptal enterocytes in newborn pigs, and no staining was observed in the tissue sections obtained from older piglets or in trypsin-treated sections. Conversely, with MAb G43 as the primary antibody, staining of the villous enterocytes was observed in both the newborn (Fig. 3B) and the older piglets. No staining of the cryptal enterocytes was seen. No specific staining occurred in any of the sections when the anti-*R. equi* MAb was used as the primary antibody or when the primary antibody was omitted.

Determination of distribution of APN and the 200-kDa protein on porcine enterocytes by FACS. Enterocyte fractions were collected from the jejuna of two newborn pigs, and the cells were labeled with either the anti-APN MAb G43 or MAb 166, directed against the 200-kDa protein, and analyzed by FACS. The percentage of cells in each fraction labeled by each antibody is listed in Table 2. The highest percentage of labeled cells was always found in fraction I, which was collected from the tips of the villi, and while relatively few cells in the lower fractions were labeled by MAb 166, relatively larger numbers

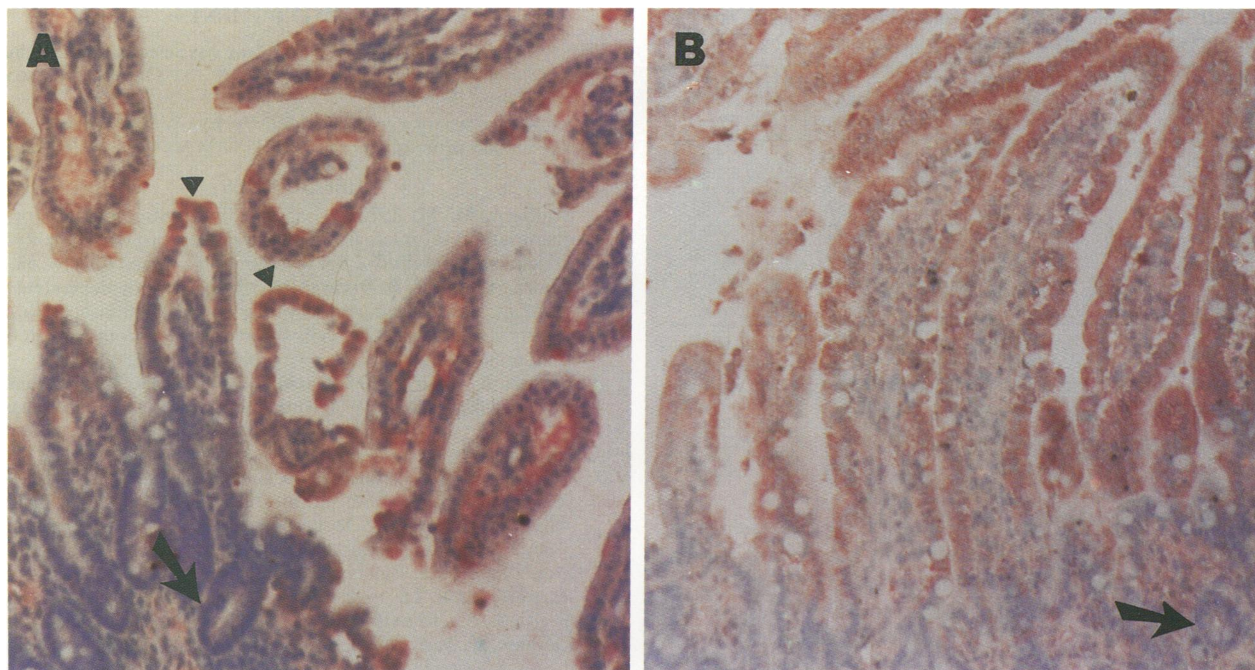

FIG. 3. Immunostaining of jejunal tissue sections from a newborn pig with the anti-200-kDa protein MAb 166 (A) or the anti-APN MAb G43 (B). MAb 166 stained islands of cells in the upper villi (arrowheads), while the crypts (arrow) remained unstained. In contrast, MAb G43 stained all of the villous epithelial cells uniformly, while the crypts (arrow) remained unstained.

of cells in each fraction were labeled by MAb G43. The distribution of the 200-kDa protein corresponded with the previously described (23) binding of TGEV to the same enterocyte fractions. FACS analysis of ST cells revealed labeling of virtually 100% of the cells with each antibody. In ST cells, the intensity of APN staining was about 15 times higher than the intensity of staining of the 200-kDa protein, whereas in villous enterocytes, the APN staining was only about five times more intense than the 200-kDa protein staining.

DISCUSSION

The high degree of tropism of TGEV for the villous enterocytes of newborn pigs is well established (12, 13) and has been suggested as a factor in the age sensitivity of newborn pigs to the virus (22). It has also been suggested (21) that the cessation of mucosal uptake of macromolecules in the intestine by 19 days after birth and the increased resistance of piglets to enteric virus infections at this time are dependent on the final disappearance of cells of fetal origin from the intestine. As a result of early studies of porcine neonatal villous enterocytes (22), it has been suggested that the rather extensive system of microcanaliculi which characterizes these cells might facilitate virus uptake and virus production. Moreover, it has been shown more recently that the binding of TGEV to the villous enterocytes of newborn pigs and to ST cells was significantly higher than the binding of virus to all other enterocyte fractions from both newborn and weaned piglets (23). The high binding was saturable, indicating the presence of specific binding sites on the cells.

APN has been identified as a major receptor for TGEV (3); however, in the present study, it is shown that the distribution of APN, as determined by assays of enzymatic activity and by immunohistochemistry and FACS with an anti-APN MAb, failed to correspond with the previously described (23) virus-binding characteristics of porcine enterocytes. Since APN was found to be present on large numbers of enterocytes from both newborn and older piglets and was found to be in high amounts, it was somewhat puzzling that the binding of TGEV to the enterocytes from older piglets was not saturable (23). Possibly, the specific affinity of APN for TGEV is rather low and, therefore, not detectable by the type of assay which was used. We concluded that the saturable virus binding by villous enterocytes from newborn pigs was not due to APN but to an additional receptor for the virus.

To attempt to identify an additional receptor for TGEV on porcine neonatal villous enterocytes, we used plasma membranes derived from these cells for the preparation of MAbs which would block virus binding and replication in ST cells. Two of these MAbs immunoprecipitated a 200-kDa nonglycosylated protein from ST cell lysates, and we believe that this may represent a second receptor or binding factor for TGEV. The results of FACS indicated that the ratio of APN to the 200-kDA virus-binding protein is lower in newborn enterocytes than it is in ST cells. This may be why we were successful in producing MAbs which were reactive against the relatively sparse 200-kDa protein. Of the four MAbs which blocked viral replication, one was directed against APN.

While the anti-APN MAb stained most of the villous enterocytes in the tissue sections, only patches of enterocytes, which were located mainly on the upper part of the villi of the jejuna of newborn pigs, were stained with the anti-200-kDa protein MAb. This corresponded with the distribution of fetal cells on the villi of newborn pigs (21) and with the distribution of cells stained with anti-TGEV antibodies in sections of the jejuna of pigs infected with the virus (7), although others have found TGEV antigen to be more widely distributed throughout the villous enterocytes (12). The lack of specific staining of the 200-kDa protein in sections which had been treated with trypsin suggested that the protein is expressed on the cell

TABLE 2. Distribution of 200-kDa and APN molecules in porcine enterocyte fractions from newborn pigs determined by FACS

Enterocyte fraction[a]	MAb 166 (%)		MAb G43 (%)	
	Pig no. 1	Pig no. 2	Pig no. 1	Pig no. 2
I	27	58	55	56
II	9	30	44	30
III	7	7	39	13
IV	8	8	42	17
V	5	8	39	19
VI	2	8	31	18
VII	5	9	38	27

[a] Fractions I and II were derived from the upper third of the villus, fractions III and IV were derived from the middle third, fractions V and VI were derived from the lower third, and fraction VII contained cryptal enterocytes. Values are percentages of cells labeled with the indicated antibodies.

surface. By FACS, the distribution of cells expressing the 200-kDa protein corresponded well with the pattern of high, saturable virus binding previously obtained (23).

On the basis of the findings reported in the present paper, we postulate the existence of two receptors which contribute additively to the binding of TGEV: the previously described (3) APN, which seems to be widely distributed on enterocytes and probably on other tissues, irrespective of age; and a second 200-kDa protein receptor which is restricted to the villous enterocytes of newborn pigs and which may be a major factor in determining the high level of susceptibility of newborn pigs to TGEV. More than one receptor has been identified in relation to the tissue tropism of several other viruses, including mouse hepatitis virus (10), herpes simplex virus (18), rabies virus (6) and bovine viral diarrhea virus (27). In the case of poliovirus (19), in addition to the virus receptor, a protein has been identified which, while not functioning as a receptor, is critical for virus attachment. This protein is believed to be involved in the determination of tissue specificity. However, TGEV may be the first virus for which a second receptor with a specific role in relation to age susceptibility has been identified. It has been proposed elsewhere (16) that the S protein of enteric strains of TGEV possesses two receptor-binding sites, while respiratory strains of the virus have only a single receptor-binding site. Our finding that the binding of TGEV, but not PRCV, to ST cells was blocked by antibodies against the putative 200-kDa receptor for TGEV seems to support this hypothesis. It would also be of interest in this context to determine whether the putative 200-kDa receptor is present in porcine respiratory tissues.

In conclusion, we suggest that the presence of a receptor or binding factor on the enterocytes of newborn pigs which has high affinity for TGEV, such as the putative 200-kDa receptor, may explain the high susceptibility of newborn pigs to the virus. This receptor may be restricted to the absorptive epithelial cells of fetal origin, in which case it would be lost when these cells are shed from the villi with increasing age. This hypothesis could also explain the observation that in infected piglets, the cells which replace the virus-infected enterocytes are relatively resistant to the virus (5), since the replacement cells would be of the adult type, lacking the high-affinity receptor for TGEV. Conversely, the more widespread, lower-affinity APN receptor would contribute to the survival of TGEV in the porcine population by facilitating low-grade replication of the virus in pigs of all ages.

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