Transmissible Gastroenteritis (TGE) of Swine: Survivor Selection of TGE Virus Mutants in Stomach Juice of Adult Pigs

By JEAN-MARIE AYNAUD,* TIEN DUNG NGUYEN,† ELISABETH BOTTREAU, ANDRE BRUN‡, AND PHILIPPE VANNIER§
INRA, Laboratoire de Pathologie Porcine, 37380 Nouzilly, France

(Accepted 24 May 1985)

SUMMARY
Two transmissible gastroenteritis (TGE) virus mutants (188-SG and 152-SG) were obtained from a low-passage virus strain (D-52) by 188 and 152 cycles of stomach juice treatment and multiplication in cell culture. Compared to the high-passage Purdue-115 and the original D-52 strains, these mutants were more stable at pH 2.0, more resistant to pepsin and trypsin, and characterized by a small plaque phenotype. In vivo, the two mutants were not found to be virulent for 4-day-old piglets and sows after oral inoculation. To test induction of lactogenic immunity, the 188-SG mutant was administered orally to pregnant sows (6 or 7 weeks before parturition) followed by one intramuscular booster (1 week before parturition). After challenge with virulent TGE virus, piglet mortality 7 days after exposure was reduced (to 22%) as compared to the death rate in piglets from control sows (91%).

INTRODUCTION
Transmissible gastroenteritis (TGE) is a highly contagious infection of swine caused by a coronavirus which is considered to be a major cause of death in newborn piglets (Bohl, 1981; Garwes, 1982). Passive protection of newborn piglets against TGE by vaccination of the pregnant sow with cell culture-adapted virus has proven unsuccessful (Saif & Bohl, 1981). Moreover, lack of seroconversion of sows has been recorded after oral vaccination with both low- (Saif & Bohl, 1979) and high-passaged virus (Voets et al., 1980). Induction of lactogenic immunity against TGE would be strongly dependent on an adequate antigenic stimulation of the gut (Saif & Bohl, 1981; Bachmann & Hess, 1982). The low stability of cell culture-adapted TGE virus strains in gastric and gut juices (Aynaud & Bottreau, 1984), which may destroy the virus during transit through the gut, is probably responsible for oral vaccination failures. With the aim of selecting mutants which would survive gastric passage, TGE virus was exposed to stomach juice and the survivors were grown in cell culture.

This paper describes the properties of two such mutants in vitro and in vivo as compared to those of the original low-passage D-52 strain and the high-passage Purdue-115 strain.

METHODS

Cells. RP, TG and RP. D are two pig kidney cell lines which have been described elsewhere (Laude et al., 1981). The third pig kidney cell line (RP. J) has been established in our laboratory by J. Gelfi. The swine testis ST cell line (McClurkin & Norman, 1966) was supplied by Dr E. H. Bohl (Wooster, Ohio, U.S.A.). Minimal essential medium (MEM) supplemented with 10% foetal calf serum was used for cell growth.

Viruses. The original virulent D-52 strain which had been isolated from an acute case of TGE was passaged five times in RP. TG cells. Purdue-115 is a high-passage strain; 152-SG and 188-SG are high-passage strains obtained in our laboratory by serial cycles of survivor selection in gastric juice. Virus suspensions were prepared by inoculating RP. D or RP. J cell monolayer cultures maintained in MEM without serum. Infected cultures were

* Present address: National Institute of Veterinary Research, Bach-Mai, Hanoi, Vietnam.
‡ Present address: Rhône-Mérieux, Laboratoire IFFA, 254, rue Marcel Mérieux, 69007 Lyon, France.
§ Present address: Station de Pathologie Porcine, 22440 Ploufragan, France.
harvested at the first sign of c.p.e. (about 15 h post-infection) and stored at -70 °C. The infectivity titres (per ml) were: strain D-52, 5 × 10^7; strain Purdue-115, 1.6 × 10^8; strain 152-SG, 6.6 × 10^6; strain 188-SG, 6.6 × 10^6. The virulent Gep-II strain of TGE virus was isolated from a field outbreak. A virulent virus stock (about 10^6 LD_50/ml) was prepared from the contents of the small intestine of colostrum-deprived newborn piglets inoculated with the Gep-II strain, harvesting 24 h post-inoculation. The inoculum was shown to be free of rotavirus by a virus enzyme-linked cell immunosassay (Grom & Bernard, 1985).

**Plaque assay.** Two- to 3-day-old monolayer cultures of ST cells were produced by seeding 5 × 10^3 cells per 30 mm container (six-well trays). The cultures were inoculated with an appropriate TGE virus dilution, and 2 ml MEM supplemented with 2% calf serum and 1% agarose (Indubiose) were added. Plaques were counted by neutral red staining following incubation at 37 to 38 °C in 5-5% CO_2 for 48 h.

**One-step growth curve.** ST cell cultures were inoculated at a m.o.i. of 5 to 8 p.f.u./cell. After incubation for 60 min at 38 °C, the cells were rinsed three times. Vials containing 4 × 10^6 infected cells in 8 ml MEM with 5% calf serum were incubated in a water-bath at 38 °C. Samples were obtained at the times indicated, diluted (1:10) in MEM with 2% calf serum and stored at -70 °C until titration.

**pH stability tests.** The virus was diluted 1:10 (v/v) in McIlvaine's buffer (0.1 M, pH 3.0 and 7.0) and in HCl–glycine buffer (0.1 M, pH 2.0). The pH value was checked before and after incubation of the virus suspension at 38 °C. At designated times, samples were taken and immediately diluted in ice-cold MEM with 20% calf serum. Residual infectivity was titrated immediately.

**Stability against proteolytic enzymes.** The virus was diluted 1:10 (v/v) in McIlvaine's buffer (0.1 M, pH 3.0) containing pepsin, or in MEM containing trypsin, chymotrypsin, pancreatin or bromelain. The mixtures were incubated at 38 °C for 30 min (pepsin) or 60 min (other enzymes). The enzyme action was then stopped by dilution in ice-cold MEM containing 10% calf serum and residual infectivity was titrated immediately. The following enzyme preparations were used: crystallized trypsin (Sigma Type IX) from porcine pancreas, containing 15550 BAEE units/mg; crystallized a-chymotrypsin (Worthington) from bovine pancreas, containing 50 BTEE units/mg; crystallized pepsin (Sigma) from porcine stomach mucosa containing 2400 haemoglobin units/mg; pancreatin (Sigma grade VI) from porcine pancreases containing 2200 BAEE units/mg (determined by I. Lantier); bromelain (Sigma) containing 1.9 amino-nitrogen units/mg with 1 mM-dithiothreitol (DTT) (Boehringer).

**Survivor selection in tissue culture.** After 5 min incubation at 37 °C in gastric juice (pH ≤ 2.5) the surviving fraction of TGE virus strain D-52 was propagated in RP. TG monolayer cultures. After 24 to 72 h at 37 to 38 °C, the viral suspension was harvested and immediately incubated again in gastric juice. Serial passages were continued in this way in tissue culture, with intervening treatments in gastric juice. After 152 and 188 passages, plaque cloning was carried out. Isolates 152-SG and 188-SG represent two mutants of TGE virus which were isolated at the 152nd and 188th passages respectively.

**Examination of the pathogenicity of different TGE virus strains in the newborn piglet.** Eleven litters of piglets suckling seronegative gilts were used. When 4 days old, the piglets were each inoculated orally with 1 ml of virus. Blood samples were collected from piglets, 2 weeks post-inoculation, and were examined for the presence of neutralizing antibody, using a microneutralization test in RP. TG cells (Toma & Benet, 1976).

**Virus inoculation of pregnant sows and virulent challenge.** Animals experimentally inoculated with TGE virus were housed in isolation units. Strain 188-SG (5 × 10^7 p.f.u.) in 300 ml of McIlvaine's buffer (0.025 M, pH 4.0) was administered per os to seven pregnant, TGE-negative, susceptible fasting sows 42 to 49 days before parturition. An intramuscular booster injection (5 × 10^7 p.f.u.) of strain 188-SG was administered 7 to 15 days before farrowing. All the suckling piglets when 4 to 9 days old were challenged with 1 ml of the virulent Gep-II strain containing 1000 LD_50 given orally. As control, litters from five uninoculated sows were challenged with the virus in the same way as above. Clinical signs and mortality rate of the piglets were noted.

## RESULTS

**Stability to low pH and digestive enzymes**

**Low pH**
Whatever strain was tested, TGE virus infectivity was slightly affected after 30 min at pH 3.0 (titre reduction by 0.1 to 0.5 log_{10} units), but at pH 2.0 clear-cut differences were observed between TGE virus strains (Fig. 1). The D-52 and Purdue-115 strains were highly sensitive (titre reductions 3.4 and 4.4 log_{10} units, respectively). In contrast, strains 152-SG and 188-SG exhibited a moderate sensitivity to acid conditions (titre reductions of 2.3 and 2.0 log_{10} units, respectively).

**Pepsin**
The level of virus inactivation by pepsin was related to the enzyme concentration. Virus strains D-52 and Purdue-115 were labile (titre reductions of 1.8 and 2.0 log_{10} units, respectively),
whereas strains 188-SG and 152-SG were more resistant (titre reductions of 0.8 and 1.3 log_{10} units) (Fig. 2a).

**Trypsin**

The level of virus inactivation by trypsin was related to enzyme concentration and clear-cut differences were observed between virus strains. Virus strains D-52 and Purdue-115 were labile (titre reductions 2.7 and 3.0 log_{10} units, respectively). On the other hand, the 188-SG and 152-SG strains were more resistant (titre reductions of 0.8 and 1.3 log_{10} units, respectively) (Fig. 2b).

**Alpha-chymotrypsin**

TGE virus infectivity was also sensitive to α-chymotrypsin and dependent on enzyme concentration. However, differences between TGE virus strains were not evident (Fig. 3a).

**Pancreatin**

Irrespective of the strain of TGE virus tested, no significant inactivation was recorded even at the highest concentration of pancreatin (Fig. 3b).

**DTT and bromelain**

TGE virus was sensitive to treatment with DTT (Fig. 4). Virus inactivation was dependent on the concentration of DTT and no differences were observed between the 188-SG and Purdue-115 strains. Strains D-52 and Purdue-115 were not affected by 60 min incubation at 37 °C in the
presence of bromelain and DTT (Table 1). In contrast, the titres of the strains 188-SG and 152-
SG were decreased (reductions of 2.0 and 1.7 log_{10} units, respectively).

**Plaque morphology**

The plaque morphology of the TGE virus strains varied in the different cell lines used. In ST
cell culture, the mutant strains (188-SG and 152-SG) exhibited small plaques (0.5 to 1.5 mm)
whereas the other strains exhibited normal sized plaques (2.0 to 2.5 mm). In the three swine
kidney cell lines (RP. D, RP. TG and RP. J), all the virus strains produced normal sized plaques.

**One-step growth curves**

One-step growth curves of the different TGEV strains in ST cell cultures are shown in Fig. 5.
The Purdue-115 and D-52 strains exhibited growth curve patterns similar to those obtained by
Laude et al. (1981). However, under similar conditions, the growth of strain 188-SG was found to
be restricted, showing a 2 h lag period and a reduced final virus titre. Strain 152-SG exhibited
the same pattern (data not shown).
Table 1. Comparative effects of bromelain in the presence of DTT on infectivity of four TGE virus strains.

<table>
<thead>
<tr>
<th>Virus</th>
<th>MEM</th>
<th>DTT*</th>
<th>Bromelain† and DTT*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purdue-115</td>
<td>3\times10^7</td>
<td>3\times10^7</td>
<td>1\times10^7</td>
</tr>
<tr>
<td>D-52</td>
<td>1\times10^7</td>
<td>1\times10^7</td>
<td>6\times10^6</td>
</tr>
<tr>
<td>152-SG</td>
<td>3\times10^6</td>
<td>1\times10^6</td>
<td>1\times10^5</td>
</tr>
<tr>
<td>188-SG</td>
<td>2\times10^6</td>
<td>1\times10^5</td>
<td>2\times10^4</td>
</tr>
</tbody>
</table>

* 1 mM.
† 1.3 mg/ml.

Table 2. Virulence of different TGE virus strains in newborn piglets

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of piglets</th>
<th>Intensity†</th>
<th>n‡</th>
<th>Mortality (n)</th>
<th>Antibody presence§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gep-II</td>
<td>23</td>
<td>+++</td>
<td>23</td>
<td>23</td>
<td>–</td>
</tr>
<tr>
<td>Purdue-115</td>
<td>12</td>
<td>++</td>
<td>12</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>6†</td>
<td>+</td>
<td>5</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>D-52</td>
<td>15</td>
<td>++</td>
<td>12</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>4†</td>
<td>+</td>
<td>3</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>152-SG</td>
<td>7</td>
<td>+</td>
<td>4</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>6†</td>
<td>+</td>
<td>3</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>188-SG</td>
<td>3</td>
<td>+</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3‖</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

* Virus dose: 10^3 LD_{50} per piglet for the Gep-II strain and 2 \times 10^6 to 2 \times 10^7 p.f.u. per piglet for other virus strains.
† +++, Early vomiting and diarrhoea which was acute and persistent; ++, moderate but persistent diarrhoea; +, mild and delayed diarrhoea for 1 or 2 days only; –, no clinical symptoms.
‡ n, Number positive.
§ Presence of neutralizing antibody in serum 2 weeks post-infection.
‖ Uninoculated contact piglets.

Table 3. Mortality rate of newborn piglets suckling sows immunized with the strain 188-SG after challenge exposure with virulent TGE virus

<table>
<thead>
<tr>
<th>Sows</th>
<th>Mortality of piglets*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No./total</td>
</tr>
<tr>
<td>Immunized (7)†</td>
<td>12/54</td>
</tr>
<tr>
<td>Controls, susceptible (5)†</td>
<td>32/35</td>
</tr>
</tbody>
</table>

* Seven days after challenge exposure.
† Number of sows is given in parentheses.

Experimental inoculation of newborn piglets

Clinical results are shown in Table 2. All piglets inoculated with the virulent strain Gep-II developed an early (on the 1st day post-infection) acute and persistent diarrhoea with vomiting, which is characteristic of TGE. On the other hand, after inoculation with strains 152-SG or 188-SG, piglets showed delayed and mild diarrhoea (appearing on day 5 or 6 post-infection, and lasting 24 to 46 h. Death was not observed, but all animals, inoculated and uninoculated 'contact', became seropositive in spite of the absence of diarrhoea in the latter and of the failure to isolate virus from faecal material in tissue culture.

Virus inoculation of pregnant sows

Results are presented in Table 3. Upon inoculation, clinical reactions were not observed and all seven sows became strongly seropositive. At the time of challenge exposure, high levels of
neutralizing antibody in the serum [geometric mean titre (GMT) 1/1722] and in the milk (GMT 1/332) of these sows were detected (data not shown). Piglets from inoculated sows developed moderate and delayed diarrhoea 3 or 4 days after challenge exposure. In contrast, piglets from control uninoculated sows developed early (24 h post-exposure), acute and persistent symptoms (vomiting and diarrhoea). Within 7 days after challenge exposure, the mortality rate was 22% in piglets suckling the seven inoculated sows, compared with 91% in piglets suckling the five non-inoculated sows.

DISCUSSION

In confirmation of previous reports (Mocsari, 1980; Laude et al., 1981) the present experiments demonstrate the lability of TGE virus, irrespective of the strain tested, in the physicochemical environment of the digestive tract of the adult pig. The results also demonstrate that the increased stability of TGE virus mutants isolated by a survivor selection procedure using the stomach juice of adult pigs is associated with both absence of pathogenicity for young pigs and immunogenicity for sows.

Differences in stability under acidic conditions (pH 2·0) were observed between TGE virus strains. This pH value, which has not been used by previous workers, reflects the natural pH of gastric juice of adult pigs better than pH 3·0 which is widely used as the standard pH to test acid stability of viruses. It is interesting to note that other enteric porcine viruses such as enteroviruses are by contrast unaffected by 60 min at pH 2·0, or at pH 3·0 in the presence of pepsin (data not shown).

It is also worthy of note that the TGE mutants isolated by survivor selection acquired simultaneous resistance to acidity and to pepsin and trypsin. The activity of these two proteolytic enzymes is optimum at pH 2 to 3 and at pH 7 to 8, respectively. The mechanism behind this double resistance is as yet unknown and requires further investigation. These two mutants also had an increased sensitivity to bromelain, a proteolytic enzyme which is activated by SH group reagents.

Our results indicate that the replication of the 188-SG strain in a one-step growth cycle is poor and delayed, compared to that of D-52 and Purdue-115. Moreover, the final infectivity titre of virus stock preparations of the 188-SG strain is always tenfold lower. The reason for this difference is unknown and needs further research. In any case, the poor and delayed replication of 188-SG could explain the small plaque size characteristic of this strain. In agreement with previous findings (Laude et al., 1981) our results suggest that the size of plaques under an agar overlay is more closely related to the single cycle yield than to the passage status of the strain (Hess & Bachmann, 1977).

The best criterion for evaluation of lactogenic immunity to TGE is considered to be protection of suckling piglets because the quantification is easy, and it is the result of a long immune process including several steps such as multiplication of virus in the intestinal epithelium, antigenic priming, synthesis of different Ig classes etc. The symptoms observed in suckling piglets after exposure (moderate diarrhoea, and 22% mortality) may well be due to the high dose of virulent virus (1000 LD₅₀) given to each piglet, compared to that used (100 LD₅₀) by previous investigators (Saif & Bohl, 1979). Therefore, we consider that this level of protection may be satisfactory and the 188-SG strain could be used as a live vaccine if this mutant proves to be genetically stable.

Previous investigators (Furuuchi et al., 1975; Hess & Bachmann, 1977) have reported that the degree of attenuation of TGE virus is related to its sensitivity to the physicochemical environment of the digestive tract. The results of the present study show clearly that it is difficult to establish such a correlation. Our two mutants which were more resistant to acidity and digestive enzymes were found to be attenuated. Consequently, we considered that the degree of attenuation and the sensitivity of TGE virus to low pH and to digestive enzymes are independently determined. Therefore, in our opinion, good passive protection (78%) after challenge was due mainly to the stability of the virus particles in the digestive tract.
We should like to express our thanks to Ph. Bernardet, J. L. Delaunay and D. Musset for their excellent technical assistance in caring for the pigs and also F. Cupi for expert secretarial work. Parts of these results were presented at an INRA meeting (Journées de la Recherche Porcine, Paris, February 1984) and at an EMBO workshop (Utrecht, May 1984).

REFERENCES


(Received 6 December 1984)