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In Vitro Differentiation and pH Sensitivity of Field and Cell Culture-Attenuated Strains of Transmissible Gastroenteritis Virus

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Characteristics of four transmissible gastroenteritis (TGE) virus field strains (Miller, Purdue, Bl, and V203) and four cell culture-attenuated strains (Purdue, SH, CKp, and Bl) were studied to find methods of differentiation between the two groups of viruses. TGE field virus strains did not replicate as well as attenuated strains at 37 C and could not be passaged serially for more than four to six passages at 33 C. There were clear differences in plaque size when the strains were compared. Field strains had average plaque sizes ranging from 3.59 to 3.15 mm, whereas attenuated strains induced plaques that were larger than 4.2 mm. Variations were observed in stability of strains at pH 3.0. Field strains and cell culture-attenuated strains CKp-270 and SH-114 were reduced in titer by about 1 $\log_{10}$. A reduction of about 3 $\log_{10}$, however, was obtained with cell culture strains B1-300 and Purdue-113.

Protection of newborn piglets against transmissible gastroenteritis (TGE) by vaccination is still an unsolved problem (5–7, 9). Immunity to TGE in these animals is known to be related directly to the presence of antibody in the intestine. Since very young piglets cannot be actively immunized against TGE before infection occurs, the protective antibody must originate from the milk of the sow. This passive immunity in the piglet is mediated mainly by immunoglobulin A antibodies (5–7, 9), and effective vaccination procedures should be directed towards the stimulation of immunoglobulin A antibody production. Although this mechanism can be initiated after oral infection of sows with field strains of TGE virus, all attempts to induce sufficient immunoglobulin A stimulation in sows with cell culture-passaged and -attenuated virus strains resulted in only limited protection or failed (1, 5).

These experiences led to the assumption that characteristics of field and cell culture-attenuated TGE virus strains differ. The present paper describes investigations on comparisons of selected properties of eight TGE virus strains.

MATERIALS AND METHODS

Virus. Four field strains and four cell culture-attenuated strains were used. TGE field virus strains were isolated in swine thyroid (ST) cell cultures directly from intestinal suspensions of infected piglets and were plaque purified three times in ST cells. They consisted of the Miller strain (kindly supplied by M. Pensaert, Gent, Belgium), piglet passage 3 after original recovery; Purdue strain (also supplied by M. Pensaert), piglet passage 10; Bl strain (3), piglet passage 2; and strain V203, isolated in our laboratory without further piglet passage.

The four cell culture-attenuated strains also were plaque purified three times in ST cell cultures and consisted of the Purdue strain (kindly supplied by M. Pensaert) after 113 passages in swine kidney cell cultures (Purdue-113), an SH strain (kindly supplied by B. E. Sheffy, Ithaca, N. Y.) (13) after 114 passages in swine kidney cells (SH-114), strain Bl after 300 passages in ST cell cultures (Bl-300), and a Hungarian CKp-strain (kindly supplied by A. Mészáros, Budapest, Hungary) (12) that had been passaged 270 times in ST cells after arrival at this laboratory (CKp-270).

Stocks of cloned virus were prepared from supernatant fluids obtained from frozen-and-thawed, infected ST cell material after low-speed centrifugation and stored in 2-ml portions at −70 C. Cell culture infectivity titers of the stock virus material were: Miller-3, 2×10^5 plaque-forming units (PFU)/ml; Purdue-10, 2.6×10^5 PFU/ml; Bl-3, 4×10^5 PFU/ml; V203, 6×10^4 PFU/ml; Purdue-113, 4.8×10^6 PFU/ml; CKp-270, 10^6 PFU/ml; SH-114, 2.2×10^5 PFU/ml; and Bl-300, 10^7 PFU/ml.

Cell cultures. Secondary ST cell cultures were used throughout the experiments. They were prepared with strains Bl-3, Bl-300, and SH-114. with minor modifications.

Virus growth and titration. A growth curve was prepared with strains B1-3, B1-300, and SH-114. Monolayer cultures were overlaid with 0.5 ml of virus suspension and left for 1 h at 22 C. To remove

nonadsorbed virus, the cultures were washed twice with phosphate-buffered saline, and after addition of virus medium cell cultures were incubated at 37 C. At 0, 1, 2, 4, 6, 8, 12, 16, 20, 24, 30, 38, and 48 h after inoculation, three infected cultures of each virus strain were frozen at −70 C, thawed, and, after centrifugation at 2,000 × *g* for 10 min, titrated for infectivity.

Growth of TGE virus strains at 33 C was investigated by the inoculation of each of four cell culture tubes with 0.25 ml of virus of the respective strain. After a 40-h incubation period, the cultures were frozen and thawed and centrifuged (2,000 × *g*, 20 min), and the supernatant was titrated for infectivity. All virus strains were passaged at least six times at 33 C.

Virus infectivity was assayed in microtest plates. Serial 10-fold dilutions in amounts of 0.025 ml were inoculated into 0.1 ml of cell suspension containing 10^5 cells, using eight wells per dilution. The plates were sealed and incubated in a CO_2 atmosphere at 37 C for 8 days. Titration end points (50% tissue culture infective doses [$TCID_{50}$]) were calculated according to the method of Kärber (14).

pH stability tests. Centrifuged cell culture TGE virus material was diluted 10-fold in McIlvaine's buffer adjusted to pH 3.0 or 7.2. pH values were measured before, during, and after the experiment. The acid-labile equine rhinovirus and the acid-stable enteric cytopathogenic bovine orphan virus were included in the test as controls.

After an incubation period of 4 h at 37 C, residual infectious virus was titrated. Samples were diluted 10-fold in cold Earle balanced salt solution, and for comparative reasons infectivity was assayed using two different techniques: a microtiter technique with reading of cytopathic effects (eight wells per dilution) and a plaque test using four petri dish cell cultures per dilution.

Plaque production. Secondary ST cells were grown in plastic petri dishes (60 by 15 mm). After development of a monolayer the cells were washed twice with prewarmed phosphate-buffered saline and inoculated with 0.5 ml of virus suspension containing 10 to 20 PFU/ml. The inoculum was removed after an adsorption time of 120 min, and 5 ml of medium containing 1% agar (Merck, Darmstadt, Germany; Merck purified) was added after an additional washing process. The petri dishes were incubated in a CO_2 atmosphere at 37 C for 40 h, and then the cell cultures were fixed and stained with a solution containing 250 ml of formalin (36%), 250 ml of methylene blue (5% solution), and 500 ml of absolute methyl alcohol. Three hours later, the agar was poured away and plaques were measured. Each virus strain was inoculated onto different batches of ST cells to confirm the reproducibility of the plaque size. At least 500 plaques of each sample were measured. Statistical evaluation of the differences in plaque sizes obtained with the strains was carried out using the t test.

RESULTS

In the growth curve (Fig. 1) a rise in virus infectivity started between 6 and 8 h postinoculation, and infectivity titers of $10^{6.25}$ $TCID_{50}$/0.025 ml were observed from 24 to 30 h of incubation at 37 C with the cell culture-attenuated strains SH-114 and Bl-300. The replication kinetics were similar with both strains. In contrast to these results, the Bl-3 field strain replicated only poorly up to infectivity titers of $10^{4.25}$ $TCID_{50}$/0.025 ml in the cell culture system used. This difference in growth was also observed between other field and cell culture-attenuated strains.

The results of viral replication at the suboptimal temperature of 33 C also revealed a clear difference between the growth of field and cell culture-attenuated TGE virus strains (Table 1). Field strains did not replicate at this temperature for more than four to six consecutive passages, depending upon the virus strain, and showed a gradual decrease of viral infectivity with each passage. From passage 4 to 6 and up no significant infectivity was observed with TGE field strains. The cell culture-adapted strains, however, replicated at 33 C. Virus titers also decreased to a certain extent up to passage 6, but infectivity titers remained in the range of between $10^{3.25}$ and $10^{4.5}$ $TCID_{50}$/0.025 ml.

Differentiation of field and cell culture-atten-

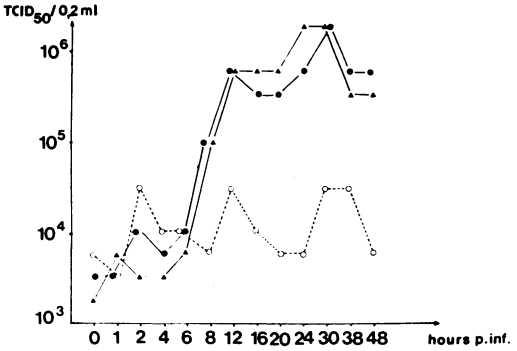

FIG. 1. *Growth curves of TGE virus field strain Bl-2 (○) and cell culture-attenuated strains SH-114 (▲) and Bl-300 (●) in secondary ST cell cultures.*

TABLE 1. *Reproduction of eight TGE virus strains at 33 C on ST cell cultures*

TGE virus strain	Titer (log_{10} $TCID_{50}$/0.2 ml) at passage:					
	1	2	3	4	5	6
Bl-2	3.0	3.5	2.1	0.5	<0.1	<0.1
Miller	2.6	2.5	2.1	0.4	0.25	0.1
Pu-10	2.4	2.5	1.0	0.1	<0.1	<0.1
V-203	3.0	3.5	2.6	0.25	0.1	<0.1
Bl-300	5.5	5.4	4.5	3.75	3.9	3.75
CKp-270	4.5	4.5	3.5	3.4	4.0	3.4
Pu-113	5.0	5.3	4.15	3.5	3.4	3.25
SH-114	4.0	4.0	3.25	3.75	4.0	4.5

uated strains was also possible by the plaque method. Field virus strains produced plaques with mean diameters varying from 2.59 to 3.15 mm, whereas high-passaged virus strains induced plaques that were always larger than 4.15 mm in size (Table 2). Cell cultures could not be older than 3 days when inoculated, in order to obtain reproducible results. Plaques were round with regular borders (Fig. 2). Mean diameters of plaques varied only a little within the group of field virus strains and cell culture-attenuated virus; however, the strains with most cell culture passages, Bl-300 and CKp-270, produced the largest plaques. Plaque sizes of field strains rarely exceeded 3.5 mm (0 to 5%), whereas 87 to 94% of all plaques of cell culture-attenuated strains measured more than 3.5 mm.

Some variation in plaque size always could be observed in the cell culture-attenuated TGE strains, although strains underwent plaque purification. Six to 13% of plaques were smaller than 3.5 mm.

All eight virus strains showed considerable differences in stability at pH 3.0. Reproducible results of pH stability of the different strains were obtained only after using the plaque method for calculation of infectivity titers. Variation of results was highest when samples were titrated by the microtiter method, calculating 50% infectivity end points. Using the plaque method, the highest variation between two experiments was 0.57 $\log_{10}$. All field strains revealed a titer reduction of about 1 $\log_{10}$ PFU after 4 h at pH 3.0. Similar results were obtained with the high-passaged strains SH-114 and CKp-270. In contrast to these results, cell culture strains Bl-300 and Purdue-113 were much more acid labile than other strains. With these two strains a titer reduction of 2.7 to 2.9 $\log_{10}$ PFU was observed (Table 3).

TABLE 2. *Plaque size and variation in diameter of eight TGE virus strains 40 h after inoculation of ST cell cultures*

Virus strains	Variation (%) in plaque diam				
	Mean diam (mm)	<2.0 mm	<3.5 mm	>3.5 mm	>5.0 mm
Bl-300	4.66	0	6	94	38
CKp-270	4.71	0	8	92	35
Pu-113	4.45	0	10	90	12
SH-114	4.21	0	13	87	8
Bl-2	2.91	10	98	2	0
Miller	2.74	11	99	1	0
Pu-10	3.15	9	95	5	0
V-203	2.59	27	100	0	0

DISCUSSION

Although it is known that in vivo biological differences exist between field and cell culture-attenuated TGE strains (4, 5, 9, 18), only little is known about the properties of these strains in vitro (15, 16). Differences between field and cell culture-attenuated strains seemed to exist only in the growth kinetics and susceptibility for certain cell cultures (5, 18). The results indicating the TGE field strains replicate only to low infectivity titers in cell cultures when compared with attenuated strains could be confirmed. Clear differences in growth were shown, in addition, at suboptimal temperatures. Field strains could not be adapted to replicate for more than four to six serial passages at 33 C, and infectivity titers gradually decreased from passage to passage. Adaptation of attenuated TGE strains to the lower incubation temperature, on the other side, was readily achieved, although infectivity titers were lower as compared with titers obtained after replication at 37 C. Whether this property of field virus strains at suboptimal temperatures could be used as marker for differentiation remains to be seen.

Further differences were observed in plaque sizes. Field strains produced plaques that measured between 2.59 and 3.15 mm, compared to plaques larger than 4.15 mm for attenuated strains. These differences were significant to the extent of $0.001 < P > 0.0001$. In contrast to these results, Bohl and Kumagai (8) could not find differences in plaque size between six field isolates and three cell culture-passaged strains that had been propagated in porcine kidney cell cultures. This contradiction can perhaps be explained by the different susceptibility of kidney and thyroid cell cultures for TGE virus.

In some respects the results on the variation of TGE strains in stability to pH 3.0 are surprising. Whereas field virus strains and the attenuated strains SH-114 and CKp-270 showed a reduction in titer of about 1 $\log_{10}$ after treatment, the cell culture-attenuated strains Bl-300 and Purdue-113 were labile at pH values of 3.0. This lability does not seem to be influenced by the passage number; however, it is a consequence of cell culture passages. It must be a specific property of the attenuated strains, since passage numbers of the stable SH-114 and labile Purdue-113 virus strains as well as the stable CKp-270 and labile Bl-300 strains are in the same range. These results are in partial disagreement with Harada et al. (13), who reported acid stability of the SH strain at pH 3.0, and Sheffy (17), who observed a reduction in

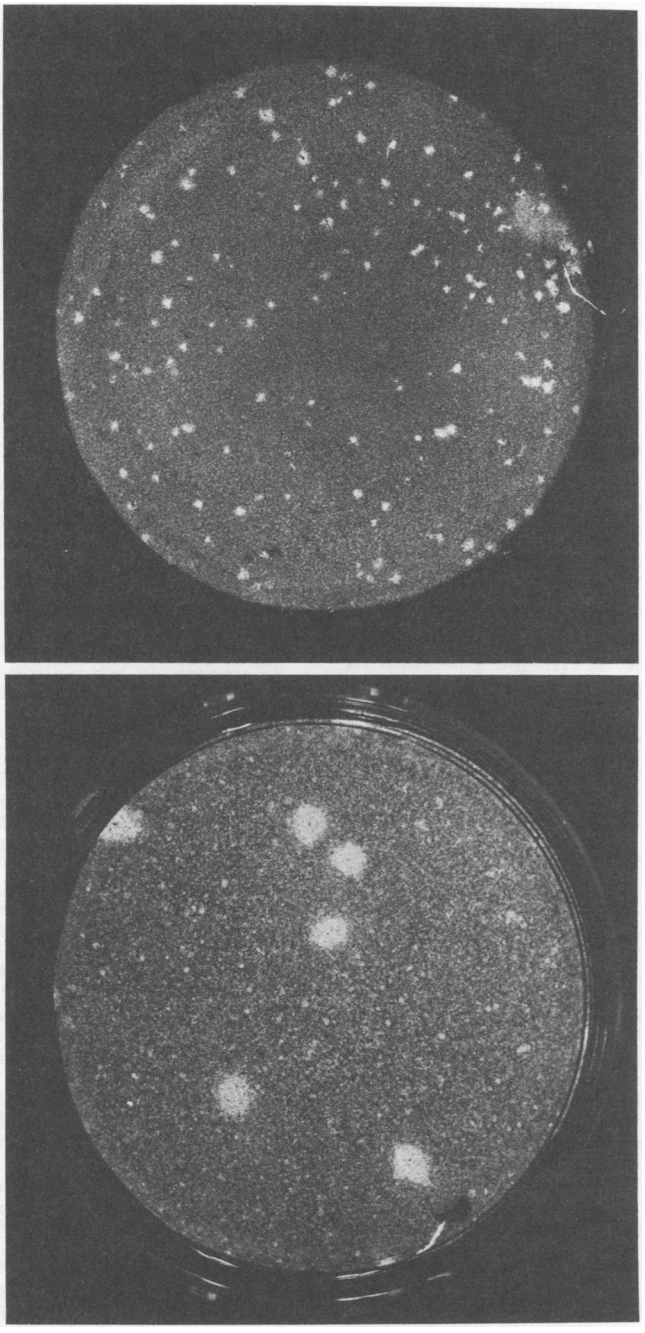

FIG. 2. *Plaque sizes and morphology of TGE virus field strain Bl-2 (top, small plaques) and the same strain after 300 cell culture passages in ST cells (Bl-300; bottom, large plaques).*

titer of about 2 $\log_{10}$ with the same strain. The discrepancies can be explained by different test procedures and frequently occur when test virus titrations are carried out without using the plaque method shown in this paper and recently by Cowan and Hitchner (10).

The observed variation in pH stability seems, however, to be a common property of coronavi-

TABLE 3. *pH stability test on eight TGE virus strains (McIlvaine's buffer)*

TGE virus strains	Titer (log_{10} PFU/0.5 ml)					
	Trial 1			Trial 2		
	pH 7.2	pH 3.0	Titer reduction	pH 7.2	pH 3.0	Titer reduction
Bl-2	5.0	3.93	1.07	5.25	4.28	0.97
Miller	5.08	3.92	1.16	4.81	3.78	1.03
Pu-10	4.98	4.0	0.98	4.99	3.86	1.13
V-203	4.18	3.1	1.08	3.52	2.65	0.87
Bl-300	6.08	3.31	2.77	6.61	3.71	2.9
CKp-270	5.23	4.36	0.87	5.74	5.44	0.3
Pu-113	5.44	2.78	2.66	6.32	3.59	2.73
SH-114	5.26	4.40	0.86	5.72	4.71	1.01
ECBO virus[a]	5.37	5.12	0.25	5.5	5.37	0.13
Rhinovirus (equine)[a]	5.12	0.25	4.87	5.25	0.5	4.75

[a] Acid-labile and acid-stable virus control. ECBO virus, Enteric cytopathogenic bovine orphan virus.

ruses, since avian infectious bronchitis virus displays a similar variability (10). Whether this variation in pH stability affects the passage of TGE virus through the stomach of pigs, where very low pH values were measured (2, 11), and consequently has an effect on the virus replication in the intestine in vivo must be shown in further experiments.

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