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Characterization of the TO Strains of Theiler's Mouse Encephalomyelitis Viruses

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Theiler's mouse encephalomyelitis virus isolates from the central nervous systems of spontaneously paralyzed mice and stools of asymptomatic mice resemble Theiler's original virus isolates. In this study four such strains were adapted by blind subpassage to replicate and to produce cytopathic effect in cell culture. These viruses were then found to be closely related to each other and to GDVII virus by cross-neutralization and to form small plaques. Bovine serum was found to contain cross-reacting antibodies to these viruses.

The Theiler's mouse encephalomyelitis viruses (TMEV) are picornaviruses which cause widespread enteric infection and, on occasion, spontaneous paralysis of colony-bred mice. TMEV isolates have been recovered from the intestinal contents of normal mice, from the central nervous systems (CNS) of paralyzed mice (13, 15) and, rarely, from the CNS of encephalitic mice (8, 16). Two groups of TMEV are definable: (i) isolates from CNS of spontaneously paralyzed mice and stools of asymptomatic animals resemble Theiler's original (TO) isolates because they cause flaccid paralysis, and (ii) several more neurovirulent strains, which appear to form a second group, cause encephalitis.

There are no studies available on the serological relationships of the various strains of TMEV, using monospecific antisera. In part, this stems from the inability to propagate the TO strains in the tissue culture. This paper describes methods of adapting four TO strains to cell culture, the serological relationships of these TO strains to each other and GDVII virus, and the discovery of neutralizing antibodies in bovine sera which cross-react with TMEV.

Plaque-purified GDVII virus grown in BHK-21 cells was obtained from L. Sturman in Albany, N.Y. (12). Mouse brain stocks of the TO strains were obtained from the following sources: DA virus from the Massachusetts State Department of Health, Boston, TO4 virus from L. Sturman, WW virus from D. Gilden and S. Wroblewska in Philadelphia, Pa., and Yale virus from the American Type Culture Collection, Rockville, Md. Stocks of these TO strains were prepared from the first to third suckling mouse brain passages as 10 or 20% clarified homogenates of brain. Encephalomyocarditis virus was obtained from K. Takemoto, Bethesda, Md. L929 cells were purchased commercially, and primary baby mouse kidney (BMK) cells were prepared from 10-day-old mice by a standard trypsin dispersion procedure (10). L929 and BMK cells were maintained in Eagle minimum essential medium containing 0.1 mM glutamine, 100 μg of streptomycin, and 100 U of penicillin per ml, and 1% agamma fetal bovine serum.

Neutralizing antibodies were determined by a standard plaque reduction method as described previously (9). For hemagglutination, 50 μl of human type O erythrocytes was mixed with an equal volume of twofold dilutions of virus in phosphate-buffered saline (PBS) containing 0.5% bovine plasma, fraction V (Armour Pharmaceutical Co., Chicago, Ill.). After a 2-h incubation at 4°C, the end point was recorded as the highest dilution at which strong partial hemagglutination occurred.

Immunoglobulins were precipitated from bovine serum by adding ammonium sulfate to give 50% saturation. The precipitate and supernatant were dialyzed extensively against PBS (pH 7.4). The immunoglobulin G (IgG) fraction from bovine serum was obtained by diethylaminoethylcellulose chromatography, using a tris(hydroxymethyl)aminomethane-phosphate (0.01 M phosphate [pH 8.4] to 0.3 M phosphate [pH 4.5] continuous gradient (3). The effluent IgG fractions were pooled, concentrated, and dialyzed exhaustively against PBS. Antigenic purity of the IgG was determined by immunoelectrophoretic and Ouchterlony analysis with use of rabbit antiserum against bovine whole serum and IgG (Cappel Laboratories, Inc., Downingtown, Pa.). For 2-mercaptoethanol extraction, 0.5 ml of undiluted bovine serum was mixed with 0.5 ml of 0.2 M 2-mercaptoethanol in PBS (pH 7.4) and with PBS alone for control. After 1 h at 24°C and 16 h at 4°C, an equal volume of 1 M iodo-

acetamide in 50 mM tris(hydroxymethyl)-aminomethane-NaCl buffer (pH 8.0) was added to the mixture. The samples were held at 4°C for 30 min and then dialyzed against PBS.

Because brain-derived TO virus stocks have not produced cytopathic effect (CPE) when grown directly to tissue culture cells, four TO isolates were serially subcultured in BMK or L929 cells. At 5- to 7-day intervals, cultures were frozen and thawed three times and the clarified supernatants were inoculated onto new monolayers. All TO isolates were successfully adapted to produce CPE (Table 1). For DA virus, isolated in cell culture in 1973, the passage scheme was somewhat involved, but it generally followed a previously described method (4). Typical picornavirus CPE appeared in the sixth BMK cell passage after an intervening passage in suckling mouse brain. In contrast, TO4, WW, and Yale strains recently produced CPE after only a few blind subpassages.

All the TO strains produced small plaques, with those of DA and Yale strains predominantly 1 mm in diameter and WW and TO4 plaques <0.2 mm in diameter. In contrast, the GDVII strain of TMEV produced both small and large plaques. The small plaques were similar in size to the TO plaques, whereas the large ones were approximately 3 to 5 mm in diameter. Bactoagar (Difco Laboratories, Detroit, Mich.) was used exclusively in plaque experiments; however, preliminary experiments did show that plaque size was similar whether Noble agar, Ionagar, or Seakem agarose was used. All TO strains were found to hemagglutinate human type O erythrocytes, and the number of plaque-forming units per HA was similar for these strains as well as for GDVII virus (Table 1).

The serological relationships of the TMEV strains were studied by raising antisera in guinea pigs. Guinea pigs injected intraperitoneally with 10^7 plaque-forming units of each TMEV strain were bled 7 days later. All five TMEV strains were found to be closely related by standard neutralization testing (Table 2). Thus, no serological subgroups exist, as is the case for human polioviruses.

Because the presence of antibodies or inhibitors in bovine serum used in our media may have interfered with the adaptation of TMEV to cell culture, different lots of commercial bovine sera were tested for neutralizing activity. The sera were heat inactivated at 56°C for 30 min. Because all the TMEV strains were similar serologically, the GDVII serotype was used in the neutralization assay. Seventeen of 23 specimens had neutralizing activity at a dilution of at least 1:4 (Table 3). All but one of the sera with activity had relatively low titers. The evidence that indicated that this neutralizing activity was indeed due to antibody and not to a nonspecific inhibiting factor is summarized in Table 4. The activity was virus specific since unfractionated serum neutralized GDVII virus, but not an unrelated picornavirus, encephalomyocarditis virus. Proof that the neutralizing activity was contained in the immunoglobulin portion of the serum came from studies showing that the precipitate from addition of 50% ammonium sulfate to serum to 50% saturation and the IgG fraction from the DEAE-cellulose column neutralized GDVII virus.

Thus, the present study has demonstrated that the TO strains of TMEV are easily adapted to replicate and produce CPE in primary BMK and L929 cells. In addition, it has been shown that all strains of this group of viruses share a

TABLE 1. *Characteristics of cell culture-adapted TO strains of TMEV*

Virus	Passage history[a]	Titer[b]	Plaque-forming units $\times 10^3$/HA	Plaque size (mm)
DA	BMK-4, SMB-1, BMK-1	$10^{8.0}$	5	1
TO4	BMK-2	$10^{7.0}$	4	P[c]
WW	BMK-3	$10^{7.1}$	2.5	P
Yale	L929-2	$10^{7.2}$	5	1

[a] Number of subpassages until CPE developed.
[b] Plaque-forming units per milliliter of virus stocks.
[c] P, Pinpoint plaques (less than 0.2 mm in diameter).

TABLE 2. *Serological relationship of TMEV by cross-neutralization*

Virus	Antiserum[a]				
	GDVII	DA	TO4	WW	Yale
GDVII	128[b]	128	128	64	128
DA	32	64	32	32	128
TO4	64	32	64	64	64
WW	64	32	32	64	64
Yale	64	64	32	64	128

[a] Guinea pig sera obtained 7 days after intraperitoneal inoculation.
[b] Reciprocal of dilution that inhibited 50% of virus plaques.

TABLE 3. *Neutralization of GDVII virus by commercial bovine sera*

Titer[a]	No. of sera tested	
	Total	No. of agamma
<4	6	1
4-16	16	2
32	1	0

[a] Reciprocal of dilution that inhibited 50% of virus plaques.

TABLE 4. *Characteristics of neutralizing activity in commercial bovine sera*

Serum treatment	Neutralization of:	
	TMEV	Encephalomyocarditis virus
1. Unfractionated	+[a]	−
2. Supernatant after 50% SAS[b] precipitation	−	
Precipitate after 50% SAS[b] precipitation	+	
3. After 2-mercaptoethanol treatment	+	
4. Diethylaminoethyl-cellulose column IgG fraction	+	

[a] +, Inhibited 50% of virus plaques in standard neutralizing antibody assay; −, did not inhibit 50% of virus plaques in standard neutralizing antibody assay.
[b] SAS, Saturated ammonium sulfate.

close serological relationship. The adaptation of these viruses was accomplished to enable this serological characterization and as a necessary step for future investigation, since the brain-derived stocks do not grow directly in cell culture.

Whereas isolation of the DA strain required an involved passage scheme, TO4, WW, and Yale viruses subsequently produced CPE after limited blind subpassages (Table 1). After adaptation of DA virus, we found that bovine serum neutralized the Theiler's group of viruses in a standard antibody assay. This neutralizing activity appears to be a common property of bovine sera, since it was present in approximately two-thirds of commercial lots of serum tested (Table 3). Furthermore, this activity was shown to be due to antibody, specifically IgG, and not to a nonspecific inhibitor (Table 4). The presence of antibody capable of reacting with TMEV in the media might hinder isolation of a virus which spreads from cell to cell exclusively through the extracellular milieu. The discovery of antibody in bovine serum which neutralizes TMEV suggests that a bovine enterovirus exists which cross-reacts with TMEV; the results also indicate that this virus causes widespread infection in cows. There is precedent for cross-reaction between viruses of the same genus but indigenous to different animal species, including the enteroviruses. Graves recently showed that swine vesicular disease virus is neutralized by antiserum to coxsackie B5 virus, a human enterovirus (5). As yet, specific bovine enteroviruses have not been examined for a possible serological relationship to TMEV.

Various TMEV strains have been reported to be interrelated on the basis of mouse neutralization (11, 14) and hemagglutination-inhibition tests (1). However, very little or no information has been provided about the preparation of the antiviral sera, or the antisera were generated by multiple immunizations in the past. Similarly, Hemelt et al. (6), who showed that GDVII and Yale viruses could not be separated by cross-neutralization, used antisera produced by four immunizations which were in effect hyperimmune. In the present study, when serum obtained 7 days after one injection of virus was used all TO strains showed a close serological relationship to each other and to GDVII virus.

Finally, some additional information is available to support the dichotomy between GDVII virus (and presumably FA virus) and the TO strains of TMEV. The two groups clearly produce plaques of different sizes. The TO viruses form small plaques, and GDVII virus forms predominantly large plaques in L-cells. Although some smaller plaques were seen in the GDVII stock, it was not possible to clone the large or the small plaques to breed true in L-cells; i.e., both gave rise to large and small plaques. The type of CNS disease following intracerebral inoculation may be another feature that differentiates the two groups of TMEV. While the pathogenesis of one of the TO strains, DA virus, has been thoroughly studied and shown to cause chronic demyelinating disease in mice (2, 7), we recently have found that TO4, WW, and Yale viruses produce a similar chronic CNS disease in outbred Swiss mice (unpublished data). Therefore, it would appear that the ability to cause demyelination may be a property common to the TO strains. In contrast, GDVII virus causes encephalitis, and animals surviving an infection do not later develop demyelination.

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