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Microadaption of Hemadsorption Inhibition for Neutralization Tests with Pig Hemagglutinating Encephalomyelitis Virus

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A microneutralization test is described, in which secondary pig thyroid cells are used and end points are determined by hemadsorption with hamster erythrocytes. The method is evaluated and compared with a macromethod, in which the fluorescent-antibody staining technique is applied for reading titers. The micromethod was found to be useful because of its sensitivity and rapidity.

In recent years, assays of neutralizing antibodies in serum after infection with pig hemagglutinating encephalomyelitis virus (HEV) have been carried out either in tube cultures of pig kidney cells, where end points have been established by measuring the presence or absence of hemagglutinins in the culture fluids (3), or in plaque reduction tests, which use hemadsorption to make the foci of infection evident (4). The fluorescent-antibody staining technique has also been used to detect virus growth in cell cultures (4, 5). For large-scale serological investigations, those methods are rather laborious and time-consuming to perform. In this paper, a microneutralization test is described, in which hemadsorption is used to detect virus growth. The test is evaluated and compared with a macromethod, in which the fluorescent-antibody staining technique is applied for reading titers.

MATERIALS AND METHODS

Virus used for animal inoculation. The HEV 2063/68 virus strain (1) was used for animal inoculation at the 17th passage level in primary pig kidney cell cultures. Infectivity titer was 10^5 mean tissue culture infective doses per ml ($TCID_{50}$/ml).

Virus used for tissue culture inoculation. The HEV 2063/68 virus was also used for tissue culture inoculation, but at the 18th passage level in primary pig kidney cell cultures.

Sera used for serum neutralization test. A pool of hyperimmune serum produced from two pigs approximately 2 months of age was used. Initially each animal was orally given 2 ml of virus suspension (see above) followed by four doses of 2 ml given intramuscularly at weekly intervals. The pigs were bled 10 days after the last injection. Sixteen sera from pigs with an unknown history of disease caused by HEV but positive for HEV neutralizing antibodies were used.

Erythrocytes for hemadsorption test. Suspensions of erythrocytes from hamsters were used at a concentration of 0.5%. The cells were either freshly collected or held packed at 4 C for no longer than 1 week before use.

Microneutralization test: (i) equipment. The microtiter equipment (Cooke Engineering Co., Alexandria, Va.) consisted of: sterile, tissue culture grade, flat-bottomed plates (model M-29-ART) containing 96 cylindrical wells of 0.4 ml each; pipettes (model M-17) standardized to deliver 0.05-ml drops; and sealing tape (model M-12).

(ii) Growth medium. A growth medium consisting of modified Hanks balanced salt solution containing 1.5 g of $NaHCO_3$ per liter, 0.5% lactalbumin hydrolysate, 0.01% yeast extract, 20% fetal bovine serum, 100 IE of penicillin per ml and 0.1 mg of dihydrostreptomycin per ml was employed.

(iii) Maintenance medium. The maintenance medium was Earle salt solution supplemented with 0.5% lactalbumin hydrolysate and containing a vitamin solution consisting of choline chloride, folic acid, nicotine amide, pantothenic acid (calcium salt), pyridoxal hydrochloride, thiamine hydrochloride (each in a concentration of 0.002 g/liter), riboflavine (0.0002 g/liter), and antibiotics as mentioned above.

(iv) Cell culture. Primary cultures of pig thyroid cells grown in milk dilution bottles were washed, and the cells were detached from the glass wall of the bottles by incubation at 37 C with an equal volume solution of trypsin and ethylenediaminetetraacetate (0.01% each), made in a phosphate-buffered saline solution free from calcium and magnesium ions. After centrifugation for 5 min at 180 $\times$ *g*, the supernatant was discarded, and the cells were suspended in growth medium to a final concentration of 3.5×10^5 to 4.0×10^5 cells/ml. The cell suspension was stirred gently until used. Secondary cultures were seeded by dispensing 1 drop (0.05 ml) of the cell suspension into each well of the plate followed by 2 drops (0.10 ml) of growth medium without serum. The plates were sealed with tape and incubated for 3 days at 37 C in an atmosphere of 5% carbon dioxide in air. At this time the cell sheets were confluent, and the cultures were used.

(v) Titration of virus. Infectivity titers of virus preparations were determined by preparing serial

10-fold dilutions, using maintenance medium as diluent. The growth medium in the wells was discarded, and the plates were blotted dry with sterile filter paper. One drop (0.05 ml) of the virus suspension was transferred to each well, five wells per dilution step, followed by two drops (0.10 ml) of maintenance medium. The plates were sealed with tape and incubated at 37 C for 4 days in an atmosphere of 5% carbon dioxide in air. Although the cytopathic effect usually could be established without difficulty by microscope examination, a hemadsorption test was set up. The maintenance medium was discarded, and 1 drop (0.05 ml) of a 0.5% suspension of erythrocytes was transferred to the wells and allowed to react for 45 min at room temperature. The cultures were then rinsed with phosphate-buffered saline two to three times until no erythrocytes were present in control wells. The results were read with a microscope. Based on the degree of hemadsorption, the cultures were designated from + to +++. Hemadsorption less than 50% was designated as +, from 50 to 100% was ++, and 100% was +++. Only those indicating at least ++ hemadsorption were counted as positive.

Sera were inactivated at 56 C for 30 min. Serial twofold dilutions were prepared using maintenance medium as diluent. Equal volume of virus suspension, containing approximately 100 $TCID_{50}$/0.05 ml, was added to each dilution. The serum-virus mixtures were incubated at room temperature for 1 h. Growth medium was removed from the plates, and 2 drops (0.10 ml) of the serum-virus mixture were added to each well, followed by 1 drop (0.05 ml) of maintenance medium. Four wells per dilution step were used. The incubation and the reading procedure were performed as mentioned above. Noninfected cell cultures and tests with normal serum diluted 1:10 served as controls.

Macroneutralization test. Monolayers of primary pig kidney cell cultures were grown on cover slips (10 by 50 mm) in Leighton tubes. A growth medium of modified Hanks balanced salt solution containing 0.75 g of $NaHCO_3$ per liter, 0.5% lactalbumin hydrolysate, 0.01% yeast extract, and 10% calf serum was used. The maintenance medium was the same as that mentioned previously.

(i) Titration of virus. Serial 10-fold dilutions of the virus suspension were made as mentioned in the description of the titration above. To each Leighton tube, a 0.10-ml virus suspension was added (five tubes per dilution step), followed by incubation at 37 C for 4 days. End points were determined by using the fluorescent-antibody staining technique (see below). Based on the proportion of the monolayer affected, readings varied from + (less than 50% of the monolayer affected) to ++ (50 to 100%) to +++ (100%), requiring a reading of at least ++ to be considered positive.

(ii) Neutralization test. Serial twofold dilutions were prepared from the inactivated sera as mentioned in the foregoing description of the neutralization test. An equal volume of virus suspension containing approximately 100 $TCID_{50}$/0.10 ml was added to each dilution. The mixtures were incubated at room temperature for 1 h, and 0.2 ml of mixture was added to each Leighton tube (four tubes per dilution) and incubated for 4 days at 37 C. End points were determined as above, by using the fluorescent-antibody staining technique. Noninfected cell cultures served as controls.

(iii) Fluorescent-antibody staining technique. The indirect antibody staining technique was applied. The serum used for conjugation was produced in rabbits by hyperimmunization with porcine immunoglobulin G fraction by the method of Ressang (6). The serum was conjugated as described by The and Feltkamp (7), except that a column packed with DE 52 cellulose (Whatman) was applied in the final isolation of immunoglobulin G. The immunoglobulin G solution was concentrated to 20 mg/ml and conjugated with fluorescein isothiocyanate at a concentration of 1 mg fluorescein isothiocyanate per 100 mg protein. Unbound fluorescein isothiocyanate was separated from the conjugate on a column of Sephadex G-25 by using phosphate-buffered saline as eluent.

Cover slips were removed from the Leighton tubes, flooded with phosphate-buffered saline, and fixed in acetone for 10 min. After drying, they were covered with a diluted hyperimmune serum (as described in sera used for serum neutralization tests above). The cover slips were then incubated at 37 C for 15 min, washed for 10 min in three changes of phosphate-buffered saline, covered with a dilution of the conjugate, and incubated for a further 30 min at 37 C in a humid chamber. After the incubation period, the cover slips were washed for 5 min in two changes of phosphate-buffered saline, mounted on slides in Elvanol, and read in the fluorescence microscope.

RESULTS

By using the micromethod, 10 duplicate titrations were performed with an identical hyperimmune serum in 10 different cell batches. The estimated standard deviation was calculated to be 0.384 (Table 1). The differences between the double titration results were not significant

TABLE 1. *Neutralizing antibody titers of a hyperimmune serum determined by duplicate titrations in ten different cell batches*[a]

Cell batch no.	Log_2ND_{50}[b]		Titer of virus controls (log_{10} $TCID_{50}$)[b]
	1	2	
1	9.82	10.32	2.10
2	10.07	10.32	2.50
3	9.57	9.32	2.50
4	10.32	10.57	1.70
5	9.82	10.57	1.90
6	9.57	9.82	2.30
7	10.32	9.82	2.10
8	10.32	10.07	2.50
9	9.57	9.32	2.10
10	9.82	10.07	2.10

[a] Mean neutralizing titer $\bar{X}$ = 9.97. Standard deviation = 0.384.

[b] Fifty percent end point calculated by the method of Spearman-Kärber (7).

(Table 2). However, the differences between titer values obtained in different cell batches on different days exceeded the limits of statistical tolerance (Table 2).

Titers obtained by the micromethod were higher than those determined by the macrotest (Tables 3 and 4). Table 3 shows 10 values obtained with the same serum, determined by the micro- and the macromethods, respectively. The differences between the values obtained by the two methods are significant ($P < 0.001$). Table 4 shows the neutralizing titers of sera from 16 different pigs, as determined by the macro- and the micromethod. The differences between values obtained by the two methods are significant ($0.005 < P < 0.01$).

The sensitivity of the infectivity titration of virus suspensions was equal in the two methods (Table 5).

DISCUSSION

The procedure for the microneutralization test described in this paper has been found to be less laborious and time-consuming than the macromethod. In addition, the micromethod shows higher sensitivity in neutralizing antibody assay. It has been necessary to grow a monolayer in the wells prior to addition of either virus or virus-serum mixtures to get acceptable and reproducible virus multiplication. This is time-consuming, but for routine investigations of secondary importance. The differences between results obtained in different cell batches on different days could be attributed to the existence of different cell types in the primary and secondary thyroid cell cultures and to the differences in viral test doses. In performing comparative neutralization tests, it may therefore be important to employ identical cell

TABLE 2. *Results of computations for analysis of variance of data given in Table 1*[a]

Source of variation	Amt of variation	Degrees of freedom	Estimated variance
Between column means ...	0.068	1	0.068
Between row means	2.10	9	0.233
Residual	0.58	9	0.064
Total	2.74	19	

[a] F Variance ratio = 0.068/0.064 = 1. F Variance ratio = 0.233/0.064 = 3.641. $0.025 < P < 0.05$.

TABLE 3. *Neutralizing antibody titers of a hyperimmune serum determined by the macro- and the micromethod*

Titer no.	Log_2ND_{50}[a]	
	Macromethod	Micromethod
1	8.32	9.57
2	7.82	9.82
3	8.32	10.32
4	9.07	9.82
5	8.57	10.32
6	8.07	10.07
7	8.07	9.57
8	8.32	9.32
9	8.82	9.82
10	8.57	10.07
Mean titer of virus controls (log_{10} $TCID_{50}$)[a]	2.26	2.22

[a] Fifty percent end point calculated by the method of Spearman-Kärber (7).

TABLE 4. *Neutralizing antibody titers of 16 different sera determined by the macro- and the micromethod*

Serum no.	Log_2ND_{50}[a]	
	Macromethod	Micromethod
1	4.82	7.57
2	7.32	8.32
3	6.52	7.07
4	7.82	9.32
5	4.57	4.82
6	5.32	7.07
7	6.07	7.32
8	6.07	6.57
9	4.57	6.82
10	4.57	6.32
11	6.57	7.82
12	9.32	8.82
13	9.57	9.07
14	5.57	6.07
15	10.32	9.82
16	9.82	9.07
Mean titer of virus controls (log_{10} $TCID_{50}$)[a]	2.00	1.96

[a] Fifty percent end point calculated by the method of Spearman-Kärber (7).

TABLE 5. *Titers of a viral suspension determined by the macro- and the micromethod*

Titer no.	Titer (log_{10} $TCID_{50}$/ml)[a]	
	Macromethod	Micromethod
1	5.70	5.60
2	5.10	6.20
3	5.30	5.00
4	5.70	5.20
5	5.30	5.60
6	5.70	5.80
7		5.60

[a] Fifty percent end point calculated by the method of Spearman-Kärber (7).

batches and virus dilutions.

The microtechnique described shows advantages, especially for large-scale titrations of virus-neutralizing antibodies, due to its rapidity and sensitivity.

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