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Minicultures of Mammalian Cells in a New Plastic Plate

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A new disposable micro tissue culture plate was developed and tested for use in virological procedures. Miniature mammalian cell cultures (minicultures) were grown in these plates. Each plate contained 96 circular cultures in flat wells (7 mm in diameter). Replicate titrations of a number of viruses were performed in various tissues. Excellent reproducibility was demonstrated. Mean infectivity titers determined by miniculture methods were generally within 0.6 $\log_{10}$/ml of macro tube titrations. Standard tissue culture assay techniques such as hemadsorption, interference titration, and microneutralization were easily carried out with this method and were very reproducible. Development of this noncytotoxic disposable micro tissue culture plate now permits the routine performance of rapid, reliable, and reproducible tissue culture tests at a very significant reduction in cost and labor.

Miniature tissue culture procedures have been reported for virus titrations and neutralization tests (3, 4, 6, 9, 11, 12). Plate-to-plate variation in cytotoxicity of the plastic could not be controlled, however, resulting in very inconsistent growth of cell cultures. Consequently, the method has had only limited acceptance. Because of the value of this approach, we asked a plastics manufacturer (Falcon Plastics, Division of BioQuest) to develop a suitable micro plate by using a plastic already found acceptable for tissue culture in petri dishes and tubes. Only then could the advantages of economy, speed, and productivity of the miniculture technique be fully realized. After almost a year of developmental work, a satisfactory plate was produced.

To determine the value of these plates, a number of studies were conducted after growth requirements of various tissues were established. Replicate virus titrations and neutralization tests were then performed with a variety of viruses and tissues. This paper describes the methods used and the results of comparative studies with the new micro plate.

MATERIALS AND METHODS

Miniature cell culture (miniculture) equipment. Plastic, disposable, micro plates (8.1 by 12.3 cm) with 96 flat-bottom circular wells (7 mm in diameter by 12 mm deep) were used for all testing procedures. These plates (MicroTest II tissue culture plates; Falcon Plastics, Division of BioQuest) are similar in size and configuration to the rigid, flat-bottom plates produced by other manufacturers [Cooke Engineering Co., Alexandria, Va. (Microtiter); Linbro Co., New Haven, Conn.]. The plastic in this new plate is processed by Falcon Plastics by the same methods found satisfactory in production of other tissue culture equipment. Micro pipette droppers (Microtiter, Cooke Engineering Co.) calibrated to deliver 0.025 and 0.05 ml were used to dispense reagents into wells. Sterilization and handling of pipettes have been previously described (4). The plates were obtained presterilized in sealed plastic bags. Nontoxic tape (MicroTest II pressure-sensitive film; Falcon Plastics, Division of BioQuest) was used to seal the plate to avoid bacterial and fungal contamination.

Tissue culture. Primary and continuous cell lines were trypsinized by standard methods (7). Table 1 gives the planting concentrations of the various cell lines, along with the time for development of confluency of cell sheets, average survival time of viable cell cultures, and the growth and maintenance media used. Antibiotics were added to all media (200 units of penicillin and 100 μg of streptomycin per ml). Glutamine (2 mM) was added to all media. The plates for most of these studies were prepared by inoculating each well with 0.2 ml of the appropriate cell suspension with the use of a 1.0-ml Cornwall syringe. The plate wells were sealed with tape, and cultures were incubated at 37 C until cell monolayers were con-

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TABLE 1. *Preparation of minicultures of various tissues*

Cell type	Planting concn per well	Days to confluency	Survival time	Growth medium	Maintenance medium
			days		
Primary					
African green monkey kidney	3×10^5	5	21	Melnick medium A[a] with 5% FCS[b] and 0.2% $NaHCO_3$	EMEM[c] with 2% FCS and 0.2% $NaHCO_3$
Rhesus monkey kidney	3×10^5	5	21	Melnick medium A with 5% FCS and 0.2% $NaHCO_3$	EMEM with 2% FCS and 0.2% $NaHCO_3$
Rabbit kidney	3×10^5	5	14	EMEM with 10% FCS and 0.14% $NaHCO_3$	EMEM with 10% FCS and 0.14% $NaHCO_3$
Human heteroploid					
HeLa	2×10^5	5	14	EMEM with 10% FCS and 0.2% $NaHCO_3$	EMEM with 2% FCS and 0.2% $NaHCO_3$
HEp-2	2×10^5	5	14	EMEM with 10% FCS and 0.2% $NaHCO_3$	EMEM with 2% FCS and 0.2% $NaHCO_3$
Human diploid					
WI-38	8×10^5	5	21	EMEM with 10% FCS and 0.2% $NaHCO_3$	EMEM with 2% FCS and 0.2% $NaHCO_3$

[a] BBL, division of BioQuest.
[b] Fetal calf serum (Flow Laboratories).
[c] Eagle's minimal essential medium (Flow Laboratories).

fluent. Medium was changed by either aspirating off the fluids with a Pasteur pipette or carefully pouring off spent medium. Fresh medium was then replaced and new tape applied.

Viruses studied. The following viruses were used: poliovirus types 1, 2, and 3; coxsackie A-9, B-1, and B-3; echo types 2 and 6; parainfluenza type 1; adenovirus types 2 and 3; rubella and herpes simplex (strains MS and VR_3).

Microtitration. After confluent cell sheets were obtained, maintenance medium was removed by aspiration with a Pasteur pipette. Serial log dilutions of virus were prepared in tubes, and 0.025 ml of each virus dilution was inoculated into three to six wells with sterile micro pipette droppers. After delivering 0.2 ml of maintenance medium into each cup with a 0.05-ml dropper, the plate was covered with new tape, sealed with pressure, and incubated at 34 C in a 5% CO_2 incubator for 5 days. The minicultures were examined for viral cytopathic effect with an inverted light microscope on days 3, 4, and 5.

Rubella infectivity titrations were accomplished by the interference technique of Sever et al. (10). After 5 days of incubation, medium was changed and cultures were challenged with 100 to 1,000 $TCID_{50}$ per 0.025 ml of coxsackie A-9. End points were then examined after 3 to 4 days of additional incubation, and titers were calculated by the Reed-Muench method (5).

Parainfluenza type 1 was titered by hemadsorption by the method of Schmidt et al. (8). Virus dilutions were dropped into plates containing monolayers of primary rhesus monkey cells. After 8 days of incubation, tape seals were carefully removed and the medium was discarded. Monolayers in each well were washed five times with 0.2 ml of phosphate-buffered saline, and 0.05 ml of a 0.5% suspension of guinea pig red cells was added to each well. Plates were sealed with tape, incubated at 4 C for 30 min, and checked for hemadsorption by inverting the plates and examining them with a standard light microscope.

Herpes simplex virus titrations were performed by the method of Pauls and Dowdle (4); 0.025 ml of primary rabbit kidney (PRK) cell suspension (300,000 cells/ml) was placed in each cup simultaneously with 0.025 ml of medium and 0.025 ml of the appropriate virus dilution. End points were read at 5 days.

Macro tube titration. Macro roller tube cultures of the above cell lines were grown and inoculated (7) with the same viruses. A volume of 0.2 ml of the appropriate virus dilution was added to well-developed cell cultures in tubes containing 1.5 ml of medium. These titrations were performed simultaneously with titrations in micro plates; end points were usually determined on day 5. Different lots of tissue, however, occasionally had to be used for the macro titrations.

RESULTS

Reproducibility of the miniculture system (Table 2) was determined by performing replicate titrations on different days with the same stock seed virus and various lots of cell cultures. The average standard deviation (SD) of all replicate tests (excluding SD values of 0) was ± 0.52 $\log_{10}$/1.0 ml. The largest SD obtained for one virus titration in one cell line was ± 0.95 $\log_{10}$/1.0 ml.

Replicate titration results obtained from macro tube cultures and minicultures showed that the

TABLE 2. *Mean titers determined by macro and micro methods for different viruses and cell lines*

Virus	Tissue[a]	Titer (log_{10}/1.0 ml)		
		Macro mean[b]	Micro mean[c]	Micro SD
Polio I	AGMK	9.2	8.6 (12)	0.58
	Rh	8.3	8.4 (15)	0.88
Polio II	AGMK	7.2	7.1 (1)	0
	Rh	7.5	8.0 (6)	0.22
Polio III	AGMK	8.4	7.8 (8)	0.59
	Rh	8.4	8.2 (11)	0.30
Coxsackie B-1	AGMK	7.2	6.0 (13)	0.67
	Rh	5.6	6.0 (13)	0.28
	WI-38	3.2	4.3 (14)	0.95
Coxsackie B-2	AGMK	7.2	6.4 (13)	0.48
	Rh	6.4	5.8 (4)	0.50
	WI-38	4.9	5.7 (14)	0.53
Coxsackie B-3	AGMK	8.2	7.2 (8)	0.45
	Rh	7.2	7.1 (14)	0
	WI-38	5.2	4.5 (5)	0.22
Coxsackie A-9	AGMK	7.2	6.4 (3)	0.57
	Rh	7.2	5.4 (3)	0.58
Echo 2	AGMK	5.2	4.2 (13)	0.28
	Rh	4.4	3.9 (13)	0.80
Echo 6	Rh	—	8.1 (6)	0
Herpes simplex (VR_3)	HeLa	8.2	7.6 (19)	0.50
	WI-38	7.0	7.1 (6)	0
Adeno 2	HEp-2	3.2	3.1 (6)	0
Adeno 3	HEp-2	6.2	7.0 (6)	0
Rubella	AGMK	3.5	3.6 (2)	0
	Rh	4.2	3.6 (2)	0
Parainfluenza 1	Rh	4.5	4.5 (4)	0

[a] AGMK, African green monkey kidney (primary); Rh, rhesus monkey kidney (primary); WI-38, human diploid fibroblast cell line; HEp-2, human heteroploid cell line; HeLa, human heteroploid cell line.

[b] Mean titer of at least three determinations.

[c] Number of repetitive titrations given in parentheses.

majority of the mean infectivity titers were within 0.6 log_{10}/1.0 ml of each other. In one case, the highest difference was 1.8 log_{10}/1.0 ml. When differences were found, the titers were generally higher in the macro assays.

PRK cell suspensions infected simultaneously with herpes simplex virus (strains VR_3 and MS) demonstrated excellent agreement between the macro and micro titrations (Table 3). Multiple titrations with this micro method showed good reproducibility and were found to be accurate with little day-to-day variation (1 SD = ±0.2 log_{10}/1.0 ml). Simultaneous titrations of these two strains of virus on well-developed PRK minicultures (planted 8 days previously) also correlated well with the titers obtained by the dropped-cell suspension technique. These titers were 0.35 to 0.50 log_{10}/ml higher than those obtained by the suspension method.

The herpes simplex quantal micro cross-neutralization test of Pauls and Dowdle (4) for determining the amount of antibody to the two strains of herpesvirus was also performed by the PRK cell suspension method with human and immunized rabbit sera. After the neutralization titers were determined, the *pN* values (loglog of virus neutralized − log of final serum end point + log of test volume) were calculated for each strain (2, 4). This calculation (Table 4) expresses the neutralization potency of an antiserum to the particular strain of herpesvirus by correcting for the final dilution of both serum and virus. Repeated determinations of the differences in the *pN* values (1) for a number of sera could be consistently shown to have antibody directed to the MS (type 2) or VR_3 (type 1) strains of herpes simplex virus. An additional set of experiments revealed that the sera could be diluted in these

TABLE 3. *Results of the macro and micro methods for herpes simplex virus strains grown by primary rabbit kidney dropped-cell suspension technique*

Strain	Titer (log_{10}/1.0 ml)		
	Macro mean[a]	Micro mean[b]	Micro SD
VR_3	7.5	7.3 (14)	0.23
MS	6.1	5.9 (12)	0.20

[a] Mean titer of four determinations.

[b] Number of repetitive titrations is given in parentheses.

TABLE 4. *Comparison of pN values for three series of micro cross-neutralization tests with two strains of herpes simplex virus*

Antiserum	Calculated *pN*		ΔpN[a]
	Strain VR_3	Strain MS	
Rabbit anti-VR_3	3.0360	2.0770	0.9590
	3.4642	2.3813	1.0829
	3.5827	2.5355	1.0472
Human anti-VR_3	2.3580	1.5885	0.7695
	2.8154	2.0911	0.7243
	2.9805	2.3921	0.5884
Rabbit anti-MS	2.7205	3.1405	−0.4200
	2.2677	3.1954	−0.9277
	2.4623	3.0938	−0.6315

[a] *pN* of strain VR_3 minus *pN* of strain MS.

new plates by the microloop method (9) without excessive cytotoxicity, and the herpes simplex virus infectivity titers and the difference in *pN* values obtained by this method were essentially the same as those obtained by the standard tube dilutions of sera with subsequent neutralization.

DISCUSSION

Multiple virus titrations were performed on minicultures of different mammalian cell lines in the new Falcon micro plate. Results demonstrated excellent reproducibility and correlation with standard tube methods for each of the many viruses tested. Although most of the macro mean titers were within 0.6 $\log_{10}$/ml of the micro mean titers, variation, when present, was greater between the macro and micro titers than among the micro titers alone. This may have been caused in part by the use of different lots of cell cultures in some of the titrations. When the same cell culture lot was used, as in the dropped-cell suspension technique with herpes simplex virus, the difference between the micro and macro methods appeared to decrease.

The cytopathic effect of each of the viruses studied was clearly observable with the micro wells under low or medium objectives in the inverted microscope. End points of viral titrations were generally much easier to determine in the plates than in tissue culture tubes. The reproducibility of virus assays in the micro plates demonstrated clearly that tissue cultures can be grown and maintained satisfactorily in all the wells of the plates with remarkably little contamination. One could therefore assume that most, if not all, tissue cultures which can be grown in glass tubes should also grow successfully as minicultures in this plate. Preliminary microneutralization tests with serial dilutions of sera performed with wire loops in these plates have been extremely promising. The availability of the disposable plates under sterile conditions eliminates the need for the extensive washing, treating, and sterilization procedures previously necessary. Also, a large number of determinations can be done on one plate, which vastly increases the speed and lowers the cost of performing the tests.

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