Simplified Method for Isolation of Cytomegalovirus and Demonstration of Frequent Viremia in Renal Transplant Patients (37701)

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Isolation of cytomegalovirus (CMV) is usually very slow. The conventional culture tube method (1) is cumbersome because of frequent manipulations connected with changes of media, microscopic observations of curved monolayers, and need for large incubator space. The use of plastic trays simplifies the manipulations and observations of cultures and reduces the required incubator space. Because of the findings of St. Jeor and Rapp (2) with CMV, indicating that pretreatment of fibroblasts with 5-iodo-2′-deoxyuridine (IUdR) shortens the incubation period of virus cytopathic effect (CPE) and enhances the sensitivity of virus detection, the efficacy of the new tray culture technique was also studied with and without the pretreatment of cells with IUdR.

Materials and Methods. Cell culture and media. The fetal tonsil strain of human embryonic fibroblasts (FTE) was kindly provided by Dr. B. Wentworth; 1×10^5 cells in 1.0 ml of MEM (Eagle's minimal essential medium) with 10% fetal calf serum (MEM FCa10) was seeded in each of 24 wells of a polystyrene cell-culture tray (FB-16-24-TC, Linbro Company). The monolayers were inoculated with specimens 1–6 days after they became confluent.

Specimens. Patients studied included surgical patients receiving multiple transfusions, renal allograft recipients, patients treated by hemodialysis, and miscellaneous other patients, as well as normal controls. Ten milliliters of blood anticoagulated by heparin (50 units) and urine were brought to the laboratory on ice 1–4 hr after collection. Urine pH was adjusted to neutrality with 2.8% bicarbonate and then filtered through a 0.45 μm Millipore filter. Blood was spun at 1500 rpm for 5 min; plasma and buffy-coat layer (the latter mixed with some red blood cells) were removed separately using Pasteur pipets. Buffy coat or several drops of red cells were suspended in 0.5 ml of MEM FCa10.

Inoculation of specimens and incubation. Urine (0.1 ml), 1 drop of buffy coat or red cell suspension, or 0.1 ml of plasma was inoculated in duplicate into undrained cell cultures. A special sealer (No. 64 PSM Linbro Company) was not used, as the cultures were incubated in a humidified CO_2 incubator. The next day, the added cells were resuspended with a Pasteur pipet and removed as completely as possible, and the monolayer was washed once with Hanks' balanced salt solution containing 0.2% fetal calf serum; 1.5 ml of MEM with 2% fetal calf serum, 10 m*M* bicarbonate, penicillin (100 U/ml), and streptomycin (100 μg/ml) was then added, and the cultures incubated at 36° in an atmosphere enriched with 2.5% CO_2. Media were changed weekly. Spent media were removed using suction through a stainless-steel cannula (Beckton-Dickinson, No. 16) sterilized by flaming. The specimens were observed once per week with an inverted microscope at 60× magnification. Each tray was incubated for 40 days, at which time the cell monolayers were stained by the May–Greenwald–Giemsa method and read with the aid of a microscope (100×).

Identification. Cytomegalovirus was identified by its slow growth on primary isolation

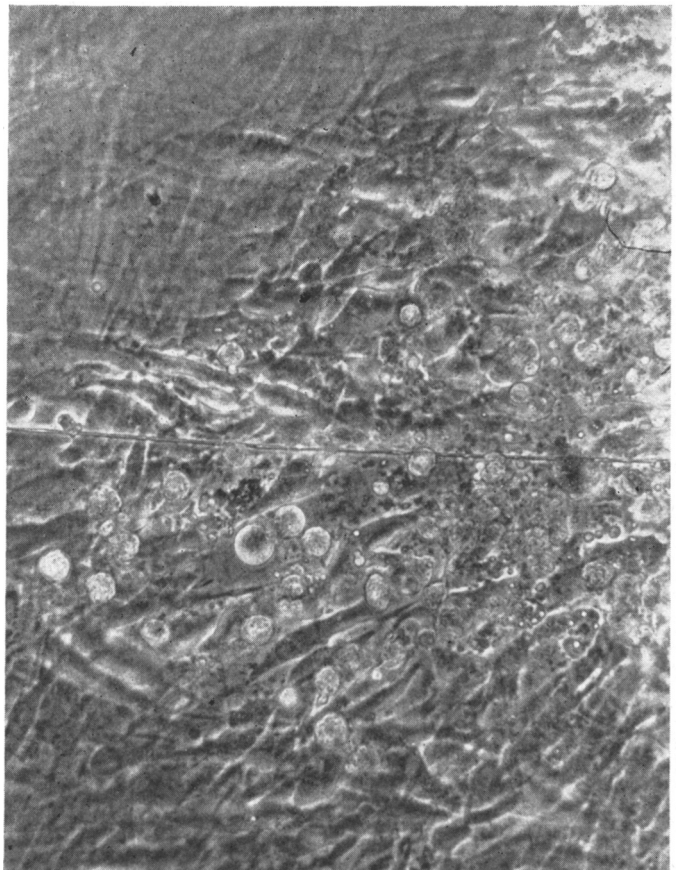

FIG. 1. Cytopathic effect produced by cytomegalovirus on human fibroblasts, appearing as rounded refractile cells.

(1–6 weeks) as well as on passage, focal cytopathic effect (CPE) on human fibroblasts appearing as a collection of rounded refractile cells (Fig. 1), and, after staining, presence of enlarged cells with typical intranuclear inclusions. On primary isolation, herpes simplex virus (HSV) produced CPE surprisingly slowly (1–6 days when isolated from urine; 14 days when isolated from blood). On passage, however, HSV CPE appeared in 1–2 days. Herpes simplex virus strains were identified by immunofluorescence in the laboratories of Dr. A. Nahmias and Dr. E. H. Lennette.

IUdR pretreatment. Trypsinized fibroblasts were resuspended in MEM FCa10 containing 100 μg/ml of IUdR, plated and incubated. Three days later, the medium was removed, and monolayers were washed for 24 hr with MEM FCa10 which was then replaced with fresh medium.

Results. Absence of cross-contamination. In order to demonstrate the absence of cross-contamination in one tray, alternate wells were inoculated with a CMV-containing urine specimen. Cytopathic effect appeared on the 11th day in all inoculated wells, and it progressed until the 40th day, while all uninoculated wells remained negative. The absence of cross-contamination was also evident from the comparison of high isolation rates in transplanted patients with low or zero rates in other patients (Table I). The specimens from all of these patients were processed together and incubated in the same trays.

Incubation period required for virus detection and virus titers. Cytomegalovirus was isolated from urine on the average about 1 week earlier than from the buffy-coat layer. Ninety percent of all positive urine specimens

TABLE I. Virus Isolation Rates Using the Plastic Tray Technique.

	Specimens							
	Urine		Buffy coat		Red cells		Other	
Patient category	No.	Positive	No.	Positive	No.	Positive	No.	Positive
Chest surgery[a]	132	1 CMV	218	2 CMV	124	1 HSV	—	—
Hemodialysis	180	1 CMV 1 HSV	306	1 CMV	116	0	—	—
Renal allograft recipients	148	56 CMV 1 HSV	178	23 CMV	5	0	—	—
Miscellaneous[b]	22	2 CMV 2 HSV	17	2 CMV	11	0	55[d]	5 HSV 2 VZV
Pregnancy	—	—	—	—	—	—	50[e]	2 CMV
Controls[c]	32	0	78	0	48	0	—	—
Total	514	60 CMV 4 HSV	797	28 CMV	304	1 HSV	105	5 HSV 2 VZV 2 CMV

[a] Patients receiving multiple transfusions.

[b] Miscellaneous: pericarditis, systemic lupus erythematosus, acute leukemia, orthopedic surgery, and various diseases suspected to be of virus origin.

[c] Controls: Medical and laboratory personnel involved in the study of CMV infections.

[d] Vesicle fluid, organ biopsies, cerebrospinal fluid, pericardial fluid, etc.

[e] Cervical swab obtained in the first trimester of normal pregnancy.

were identified in four weeks, whereas it took 5 weeks for recognition of 90% of all positive blood specimens (Table II). The number of cytopathic foci initially detected in cultures of urine was between 1 and 6, except in one urine specimen which produced 12 foci. In one-third of the specimens, only one of the two inoculated wells became positive. Twofold dilutions of two positive urine specimens were inoculated in a further attempt to titrate virus content. Cytopathic effect was found only in the undiluted and 1:2 dilutions. Thus, the virus titers in the urine of patients studied appears to be at most 1.2×10^2 plaque-forming units (PFU)/ml (12 PFU $\times$ 1/0.1 ml).

In our studies (Table I) of the blood fractions (plasma, red cells, and buffy coat), only buffy coat has yielded CMV, although HSV was isolated once from the red blood cell layer. The greatest number of CMV foci in one well was six. Assuming that each focus corresponded to an infected blood cell, the highest virus titer would be only 18 infected cells per milliliter of blood (6 $\times$ 0.1/0.03 ml).

Efficacy of the new technique compared to the conventional technique (1) and to the new technique combined with the pretreatment of cells with IUdR (Table III). Eight urine and twelve blood specimens from transplanted patients were inoculated in duplicate on either untreated (method A), or pretreated (method B) monolayers in wells, or into untreated monolayers in tubes (method C). Although CPE was recognized about one-half week earlier using the method A compared to the method B, and although the method

TABLE II. Incubation Period Required for the Detection of CMV.

Incubation period (weeks)	Cumulative positive for CMV (%)			
	Urine[a]		Blood[b]	
1–2	3	(2.8)	0	(0)
3	30	(28.1)	2	(1.4)
4	35	(32.8)	5	(3.5)
5	37	(34.6)	17	(11.8)
6	38	(35.6)	19	(13.1)

[a] 107 urine specimens were cultured.

[b] 145 blood specimens were cultured.

TABLE III. Comparison of the Efficacy of Three Techniques Using (A) Untreated Monolayers in Plastic Trays, (B) Monolayers in Plastic Trays Pretreated with IUdR, and (C) Untreated Monolayers in Culture Tubes.

Weeks after inoculation	Method A			Method B			Method C		
	Blood[a]	Urine[b]	Both	Blood[a]	Urine[b]	Both	Blood[a]	Urine[b]	Both
1	0[c]	0	0	0	0	0	0	0	0
2	1	0	1	0	0	0	1	0	1
2½	4	3	7	1	3	4	—	—	—
3	4	3	7	2	4	6	2	3	5
4	5	3	8	2	5	7	2	4	6
5	5	3	8	2	5	7	2	5	7
6	5	3	8	2	5	7	3	5	8

[a] 12 blood specimens were cultured.
[b] 8 urine specimens were cultured.
[c] Number of positive specimens.

A resulted in a slightly higher number of positive specimens than B, these differences were not statistically significant (3).

During one year, 1,720 specimens from 302 patients were processed by one technician, resulting in the isolation of 90 strains of CMV, 10 strains of HSV, and 2 strains of varicella-zoster (VZV) (Table I). Cytomegalovirus was isolated very frequently from recipients of renal allografts, infrequently from multiply transfused and other patients, and never from attending medical personnel. In 60 transplanted patients, viremia with or without viruria was detected in 18 (30%) patients and viruria alone in 12 (20%) patients. In these patients, CMV viremia was frequently associated with fever and arthralgias (Fiala, M., Payne, J., Berne, T. V., Montgomerie, J. Z., and Guze, L. B., in preparation).

Discussion. The use of plastic trays simplifies virus isolation procedure compared to the conventional technique using culture tubes (1). A single cover for the whole tray allows easy access to wells during inoculation and changing of media. The latter is facilitated by the use of a cannula and suction. The flatness of monolayers in wells permits rapid and thorough observation of virus CPE and staining of cultures *in situ*. The excellent survival of FTE cells is important, especially when culturing blood specimens which require longer incubation than urine specimens. Recently, plastic trays have been used for the titration of CMV by a microculture plaque assay (4). We have used for assay of CMV, a technique similar to the one for virus isolation. Under liquid overlay (without changing medium) cell-free CMV produced microscopic foci on the ninth day after infection. The dose-response curve of the number of foci was linear (Fiala, M., and Miyasaki, K., unpublished data).

In a limited study, virus isolation rate or the rapidity of virus detection were not significantly enhanced using the new technique compared to the tube technique. It is, however, likely that the new technique is significantly more efficient than the tube method with specimens containing low concentrations of virus (such as the buffy-coat), as one or two foci can be missed on curved monolayers, whereas they are easily seen on flat monolayers.

The virus titers in the urine and in the buffy coat of our patients were low. The virus in the urine is cell-free, as the inoculated specimens were filtered through a filter of a pore size, retaining cells. In blood, CMV was, on the other hand, associated with the buffy-coat layer, but never, in our experience, with red blood cells as was reported previously (5). Further studies to localize CMV in lymphocytes, polymorphonuclear leukocytes, or macrophages are in progress. The longer incubation period required for initial detection of virus in the buffy coat compared to the urine suggests that cell-associated virus might be inhibited by a cell repressor or by antibody. It is, therefore, surprising that pre-

treatment of cells with IUdR, which interferes with the production of cell products such as interferon (6), and which has been shown to enhance the replication of CMV (2), did not increase the isolation rate or shorten the incubation period required for virus detection.

The new technique has been shown to be very effective in the detection of CMV viremia and viruria in immunosuppressed patients. The 30% rate of viremia in renal allograft recipients found in this study is higher than the 14% incidence (one of seven patients) detected previously (7). Other viruses which grow in human fibroblasts such as HSV and varicella-zoster virus have also been isolated using our technique.

Summary. Isolation of cytomegalovirus (CMV) has been simplified using monolayers of fibroblasts in plastic cell-culture trays. The technique is at least as efficient as the conventional tube technique and may be more efficient with specimens containing low concentration of virus such as blood. Cytomegalovirus in blood has been detected only in association with the buffy-coat layer, but not with plasma or red cells. Pretreatment of fibroblasts with 5-iodo-2′-deoxyuridine (100 μg/ml) has not increased the CMV isolation rate. Using the new technique, CMV viremia was detected in 30% and CMV viruria in additional 20% of renal allograft recipients. Ninety strains of CMV, ten strains of herpes simplex, and two strains of varicella-zoster were isolated from 1,720 specimens.

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