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Recovery of Dengue Viruses from Tissues of Experimentally Infected Rhesus Monkeys

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A tissue explant culture technique for the recovery of dengue virus from experimentally infected monkey tissue is described and compared with tissue culture assay of tissue triturates and co-cultivation of trypsinized cells in cell cultures. The most efficient technique was one in which minced tissue was explanted in co-culture with dengue virus-susceptible LLC-MK2 monkey kidney cells. This technique shows promise of being useful for detection of virus in autopsy material from fatal dengue hemorrhagic fever cases.

Homogenates of tissues from normal rhesus monkeys have been found to have dengue virus inhibitory activity, the potency of the inhibitor(s) varying from organ to organ (7). These findings suggest that nonspecific inhibitors may be responsible for the neutralization of considerable amounts of dengue virus in the tissues of experimentally infected monkeys. The presence of dengue virus inhibitors in tissue homogenates also bears directly on the problem of isolation of dengue virus from human postmortem material. The difficulty encountered in isolating dengue virus from dengue hemorrhagic fever cases has been described (1, 3, 8).

In view of the occurrence in monkey tissues of nonspecific inhibitors and their variable potency in different organs, it was felt that the usual technique of trituration of tissue for virus detection by plaque assay might inaccurately reflect the distribution of dengue virus in tissues of experimentally infected monkeys. Without a reliable and accurate method for detection of dengue virus in tissues, contemplated studies of dengue pathogenesis in experimentally infected rhesus monkeys would be seriously hampered.

The present report describes the development of a tissue explant culture technique for recovery of dengue virus from infected monkey tissues and compares it with the standard trituration method and co-cultivation technique.

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MATERIALS AND METHODS

Viruses. Four dengue viruses recovered in BS-C-1 cells or suckling mice from acute phase blood specimens from dengue hemorrhagic fever patients in Thailand or the Philippines were used. These viruses are designated dengue 1 (16007), dengue 2 (16681), dengue 3 (16562), and dengue 4 (4328-S). The history and characterization of these strains have been published (4, 5). Stock virus suspensions were prepared in LLC-MK2 monkey kidney cells. The infected cell cultures were scraped from the culture bottles and suspended in phosphate-buffered saline (PBS), *p*H 7.9, containing 50% agamma calf serum and stored in 1-dram screw-cap vials at −70 C. The titer of each virus stock was determined initially and again at the time of monkey inoculation by the LLC-MK2 plaque assay technique (5).

Monkeys. Juvenile or subadult female rhesus monkeys (*Macaca mulatta*) were obtained from an animal dealer in New Delhi and were pretested for neutralizing antibody to dengue viruses. Only those demonstrably free of neutralizing antibody to all four dengue virus types in the plaque reduction neutralization test (11) were used.

Experimental infection. Dengue-susceptible or previously infected monkeys were inoculated subcutaneously with between 10^3 to 10^5 plaque-forming units of a dengue virus, bled daily for determination of viremia, and sacrificed at varying periods postinoculation. Prior to sacrifice the monkeys were kept without solid food for 24 hr, anesthetized with sodium pentothal and exsanguinated by cardiac puncture.

Trituration to tissues. Organs or portions of them were removed aseptically and placed in sterile specimen jars on ice. The tissue samples were weighed and triturated in cold mortars with pestles and sterile sand. The triturates were suspended in PBS, *p*H 7.9, containing 20% agamma calf serum to

make 20% suspensions, centrifuged at 2,400 rev/min (1,500 × *g*) for 30 min at 4 C, and the supernantant fluid was either inoculated directly onto LLC-MK2 cell monolayers for plaque assay (5) or diluted 10- or 100-fold and then inoculated for plaque assay. The inocula were allowed to adsorb onto the LLC-MK2 cells for 1.5 hr at 37 C; the mixture was then poured off and a one-stage agar overlay medium was added. The plaque bottles were incubated at 37 C for 7 days, examined for presence of plaques, and then incubated at room temperature for another 7 to 14 days before making a final plaque count.

Co-cultivation. A portion of each organ was placed in a sterile petri dish, minced with scissors, washed twice with sterile PBS, *p*H 7.2, and suspended in 20 ml of fresh 0.25% trypsin solution containing penicillin (200 μg/ml) and streptomycin. The tissue mince was agitated with a magnetic stirrer at room temperature for 45 min and the mixture was strained through several layers of sterile gauze. The trypsinized cells were collected by centrifugation at 600 rev/min (100 × *g*) for 15 min, washed once with 10 ml of culture medium, and resuspended in a small volume of fresh culture medium. The cells were counted and 0.1 ml of the suspension containing approximately 10^6 cells was inoculated onto LLC-MK2 cell monolayers in 1-oz (29.573 ml) prescription bottles. After 24 hr at 37 C, the cell sheets were washed and overlayed with agar for plaque assay, or 3.5 ml of growth medium was added to each bottle and the cultures were incubated for 7 days before the agar overlay was added for plaque assay.

Tissue explants. Tissue fragments were washed twice in PBS, *p*H 7.9, finely minced with scissors, and washed again. The washed tissue mince was placed in a 1-oz prescription bottle containing 4.0 ml of LLC-MK2 growth medium (basal medium; Eagle, 10% calf serum, glutamine, and antibiotics) and incubated at 37 C for periods up to 13 days with changes of media every 3 days, and the spent media were tested for dengue virus by standard plaque assay.

In some experiments, the minced tissue fragments were triturated after 9 days in culture and assayed for dengue virus as described above.

In several parallel experiments, tissue explants were also cultured in growth medium containing 2.5 × 10^5 LLC-MK2 cells/ml. They were treated exactly as the regular explant cultures.

RESULTS

Recovery of dengue virus from tissues of experimentally infected monkeys by the tissue explant technique. Virus recovery from explant cultures of tissues of a dengue 2-infected monkey is shown in Table 1. Explant culture medium was assayed for dengue virus after 3, 6, and 9 days of incubation at 37 C with medium changes on days 3 and 6. The efficiency of virus recovery generally increased with length of incubation period. Assay of the 3-day culture was least efficient, and assay of 6- and 9-day explant culture medium was adopted as standard procedure. The efficiency of virus recovery from tissues of a dengue 3-infected monkey was increased by continuing the explant cultures and assaying the culture medium after 13 days. Explant cultures of liver and lung from a dengue 1-infected monkey were assayed for virus at 3-day intervals for 20 days without recovering dengue virus although an adventitious agent was isolated from the lung explant culture after 13 and 16 days of incubation. No virus of any kind was recovered from explant cultures of tissues from a normal (non-dengue infected) monkey when assayed at days 6 and 9.

Trituration of the tissue mince after 9 days of culturing in growth medium and assay for dengue virus was less efficient for recovery of dengue virus than simple assay of the explant

TABLE 1. *Dengue 2 virus recovery by plaque assay from tissue explant culture medium after 3, 6, and 9 days of incubation of tissue explants at 37 C*[a]

Tissue explant[b]	Virus recovered from culture medium on day:		
	3	6	9
Right forearm skin	6[c]	>100	>100
Left forearm skin	13	67	>100
Right hind leg skin	3	41	>100
Left hind leg skin	3	>90	>100
Abdominal skin	3	6	>60
Back skin	5	>70	>100
Right axillary lymph node	0	0	0.3
Left axillary lymph node	0.3	0.3	0
Right inguinal lymph node	1	4	4
Left inguinal lymph node	0	0.6	0
Peribronchial lymph node	0.3	0	0
Thymus	0.6	3	11
Mesenteric lymph node	0	0.3	8
Esophagus	7	>70	>100
Duodenum	6	14	>50
Jejunum	1	>100	>100
Ileum	1	>80	>100
Cecum	0	>70	>100
Stomach	0	11	>100
Spleen	0	0.3	13
Adrenal	0	0	0.3
Psoas muscle	0.6	38	>100
Right submandibular gland	0.3	17	>50
Heart	0	0	1

[a] Culture medium was replaced after 3 and 6 days of incubation.

[b] Rhesus monkey H-76 was autopsied 6 days after challenge with dengue 2 and 3 months after infection with dengue 1.

[c] Plaque-forming units per 0.2 ml of explant culture medium tested by LLC-MK2 plaque assay.

culture medium on day 9 (Table 2). In only two instances was virus recovered from explant tissue triturates and not from explant medium, whereas in 11 instances virus was recovered from explant medium and not from triturates.

The addition of LLC-MK2 cells to the growth medium of tissue explant cultures slightly increased the efficiency of virus recovery from tissues of dengue 1- and dengue 2-infected monkeys (Table 3).

Comparison of explant culture and trituration of tissues for dengue virus recovery. Tissues from monkeys experimentally infected with dengue 1, 2, 3, or 4 were assayed for virus in parallel experiments by both trituration and tissue explant techniques. In each case the tissue explant technique was at least as efficient and usually more efficient than the trituration method (Table 4).

TABLE 2. *Comparison of rates of recovery of dengue virus from tissue explant culture medium and from triturates of tissue explants after 9 days of incubation in growth medium*[a]

Tissue explant	Method of assay			
	Explant medium[b]		Explant triturate[c]	
	No. positive	No. negative	No. positive	No. negative
Right forearm skin	2	0	2	0
Left forearm skin	4	0	4	0
Abdominal skin	1	2	3	0
Back skin	3	0	2	0
Right hind leg skin	1	1	1	1
Left hind leg skin	2	0	2	0
Right axillary lymph node	2	1	1	2
Left axillary lymph node	3	1	1	3
Right inguinal lymph node	3	0	0	3
Left inguinal lymph node	2	2	1	3
Peribronchial lymph node	1	2	0	3
Mesenteric lymph node	3	1	2	2
Spleen	3	1	3	1
Jejunum	2	2	2	2
Heart	1	3	1	3
Psoas muscle	1	1	1	1
Adrenal	1	2	0	3
Total	36	21	27	29

[a] Combined results of experiments with four dengue-2 infected rhesus monkeys.
[b] Total positive—63%.
[c] Total positive—48%.

TABLE 3. *Comparison of dengue virus recovery from tissues of dengue 1 (D1)- and dengue 2 (D2)-infected monkeys explanted in growth medium (GM) only and in growth medium containing LLC-MK$_2$ monkey kidney cells (GM + cells)*

Tissue	Expt. 1[a]		Expt. 2[b]		Expt. 3[c]	
	GM	GM + cells	GM	GM + cells	GM	GM + cells
Right forearm skin	+[d]	+	0	0	+	+
Left forearm skin	+	+	+	+	+	+
Right leg skin	0	–	+	+	+	+
Left leg skin	0	+	0	+	0	+
Abdominal skin	0	+	0	+	0	+
Back skin	–	–	0	0	–	–
Right axillary lymph node	–	+	0	0	+	+
Left axillary lymph node	–	+	0	0	+	+
Right inguinal lymph node	+	0	0	0	+	+
Left inguinal lymph node	+	0	+	+	0	+
Peribronchial lymph node	–	–	0	0	0	0
Mesenteric lymph node	0	0	+	+	+	–
Thymus	0	0	0	0	0	+
Esophagus	–	–	0	0	–	–
Stomach	–	–	0	0	+	+
Duodenum	–	–	+	+	0	0
Jejunum	–	–	+	+	+	+
Ileum	–	–	–	–	+	+
Cecum	–	–	–	–	0	–
Liver	0	0	0	0	0	+
Spleen	0	0	0	0	+	0
Adrenal	0	0	0	0	–	0
Kidney	0	0	0	0	–	0
Lung	0	0	0	0	0	0
Heart	0	0	0	+	0	+
Psoas muscle	0	0	0	0	0	+
Number positive	4	6	6	9	11	16
% Positive	25	35	25	38	50	73

[a] Monkey no. H-57; infection sequence D1; day 6 after infection; days of viremia, 4, 5, and 6.
[b] Monkey no. H-75; infection sequence D1; day 8 after infection; days of viremia, 5, 6, and 7.
[c] Monkey no. H-122; infection sequence D4-D3-D2 (monkey H-122 was challenged with dengue 3 six weeks after dengue 4 infection; six weeks later dengue 2 was given); day 7 after infection; days of viremia 6 and 7.
[d] Symbols: +, One or more plaques per 0.6 ml of explant culture medium in LLC-MK2 plaque assay. 0, No virus recovered. –, Not done.

Comparison of co-cultivation technique with trituration and tissue explant technique for recovery of dengue virus from tissues of experimentally infected monkeys. The co-cultivation method gave inconsistent results. It was less efficient than either the tri-

TABLE 4. *Comparison of dengue virus recovery from dengue 1 (D1)-, dengue 2 (D2)- and dengue 3 (D3)-infected monkey tissues by the trituration and explant culture techniques*

Tissue	Expt. 1[a]		Expt. 2[b]		Expt. 3[c]		Expt. 4[d]	
	Triturate	Explant	Triturate	Explant	Triturate	Explant	Triturate	Explant
Right axillary lymph node	0[e]	0	0	+	0	0	0	0
Left axillary lymph node	0	0	–	–	0	0	0	0
Right inguinal lymph node	0	0	–	–	0	0	+	+
Left inguinal lymph node	+	+	–	–	0	0	+	0
Mesenteric lymph node	0	+	0	+	0	+	0	0
Trachea	0	0	+	+	0	+	–	–
Lung	0	0	0	0	0	0	0	0
Esophagus	0	0	–	–	0	0	–	–
Stomach	0	0	0	+	0	0	–	–
Duodenum	0	+	0	0	0	0	–	–
Jejunum	0	+	0	+	0	+	0	+
Ileum	–	–	0	+	0	0	–	–
Cecum	–	–	0	0	0	0	–	–
Adrenal	0	0	0	0	0	0	0	0
Kidney	0	0	0	0	0	0	0	0
Liver	0	0	0	+	0	0	0	0
Spleen	0	0	0	+	0	0	0	+
Heart	0	+	0	+	0	0	0	0
Psoas muscle	0	0	–	–	0	+	+	0
Number positive	1	5	1	9	0	4	3	3
% Positive	6	29	7	64	0	21	23	23

[a] Monkey no. H-75; infection sequence D1; day 8 after infection; days of viremia, 5, 6, and 7.

[b] Monkey no. H-122; infection sequence D4-D3-D2; day 7 after infection; days of viremia, 5, 6, and 7.

[c] Monkey no. H-72; infection sequence D3; day 9 after infection; days of viremia, 2, 3, and 6.

[d] Monkey no. H-73; infection sequence D4-D2 (monkey H-73 was infected with dengue 2 six weeks after dengue 4 infection); day 8 after infection; days of viremia 6, 7, and 8.

[e] Symbols: +, One or more plaques per 0.6 ml of explant culture medium in LLC-MK2 plaque assay. 0, No virus recovered. –, Not done.

turation or the tissue explant technique for recovery of dengue virus from infected monkey tissues (Tables 5 and 6).

DISCUSSION

The tissue explant culture technique described has proven to be more efficient than either trituration or co-cultivation methods for recovery of dengue virus from experimentally infected rhesus monkeys. The poor results obtained with co-cultivation of trypsinized cells on monolayers of LLC-MK2 cells may have been due to loss of significant amounts of viable cells in the long process of cell dispersion and washing. Inactivation of virus may also play a role. A modified co-cultivation technique in which minced tissues were explanted in bottles seeded with dengue-susceptible LLC-MK2 cells was the most sensitive procedure so far tested. The dengue-sensitive cells were used merely as host cells for the virus to replicate in and be released into the culture medium. The culture medium, not the cells, was then assayed for virus by standard plaque assay.

The sensitivity of the explant culture technique might be due to one or more factors: (i) the virtual absence of virus inhibitors released from disrupted cells because the cells remain essentially intact and inhibitory factors are not released, (ii) elution of virus inhibitors from the minced tissues, (iii) elution of humoral antibody from tissue explants, (iv) infected cells remain viable in explanted tissue and slowly release virus into the culture medium after inhibitors or antibody have been removed, and (v) additional replication of virus takes place in infected cells in the explanted tissue increasing the chance for virus recovery.

TABLE 5. *Comparison of dengue virus recovery from primary dengue 2-infected monkeys by the trituration and co-cultivation techniques*

Tissue	Expt. 1[a]		Expt. 2[b]	
	Trituration	Co-cultivation	Trituration	CO-cultivation
Skin (inoculation site)	+[c]	+	+	0
Skin (contralateral site)	+	0	0	0
Right axillary lymph node	+	+	+	0
Left axillary lymph node	+	+	0	0
Inguinal lymph node	+	0	–	–
Mesenteric lymph node	+	0	0	0
Lung	+	+	0	0
Jejunum	+	0	0	0
Adrenal	+	0	0	0
Kidney	0	0	0	0
Liver	0	+	0	0
Spleen	0	+	0	0
Heart	0	0	0	0
Brain	0	0	–	–
Psoas muscle	0	0	0	0
Number positive	9	6	2	0
% Positive	60	40	15	0

[a] Monkey no. H-1; infection sequence D2; day 8 after infection; days of viremia 5, 6, 7, and 8.

[b] Monkey no. H-28; infection sequence D2; day 6 after infection; days of viremia 4, 5, and 6.

[c] Symbols: +, One or more plaques per 0.6 ml of explant culture medium in LLC-MK2 plaque assay. 0, No virus recovered. –, Not done.

Attempts to recover dengue virus by explant culture of previously frozen tissues, even when heavily infected, have been unsuccessful (Marchette and Halstead, *submitted for publication*). Freezing not only kills the cells, but also disrupts them, presumably releasing potent virus inhibitors which may not be eluted to noninhibiting concentrations by changes of culture medium. In certain tissues, particularly those of the digestive tract and associated organs, proteolytic enzymes (and other lipolytic substances) are present which may be responsible for inactivation of considerable amounts of virus. Group B arboviruses are known to be especially sensitive to such enzymes (2, 6, 9, 10, 13).

Presence of specific antibody in capillary beds and tissue spaces might be expected to inactivate virus released from infected cells into the explant culture medium. This may be of considerable importance in tissue taken during the convalescent phase after the viremia has disappeared. Changing the explant culture medium once or twice might remove antibody or dilute it sufficiently so that viral replication is not inhibited.

Dengue viruses remain viable for only a short time (less than 48 hr) in cell-free tissue culture medium held at 37 C (A. Diwan, *personal communication*). At least a few cells in the tissue explants remain viable for several weeks as indicated by *p*H changes in the medium and observations of cellular outgrowth when the explants are not disturbed. These observations plus the increase in virus titer with time of incubation of explanted tissues suggest replication of dengue virus in cells of infected monkey tissue explants.

Although the early work of Siler et al. (12) showed that dengue virus remained viable for at least 99 hr in serum held at refrigerator temperature, dengue viruses have only rarely been recovered from autopsy materials usually kept a similar time at cooler temperatures (8). Commonly, autopsy tissues are frozen prior to processing in the laboratory. Similarly, we have failed to recover dengue virus from monkey tissues known to contain virus, but which were frozen prior to trituration and

TABLE 6. *Comparison of dengue virus recovery from primary dengue 4-infected monkeys by co-cultivation and tissue explant techniques*

Tissue	Expt. 1[a]		Expt. 2[b]		Expt. 3[c]	
	Co-cultivation	Explant	Co-Cultivation	Explant	Co-Cultivation	Explant
Right forearm skin	0[d]	0	0	+	0	0
Left forearm skin	0	0	0	0	0	+
Abdominal skin	0	0	0	+	0	0
Right forearm subcutaneous tissue	0	0	0	+	0	0
Right axillary lymph node	0	+	0	+	0	0
Right axillary brown fat	0	+	0	0	0	0
Mesenteric lymph node	0	+	0	0	0	0
Thymus	0	+	0	0	0	0
Adrenal	0	+	0	0	0	0
Jejunum	0	0	0	0	0	+
Spleen	0	0	0	+	0	0
Leukocytes	0	0	0	0	0	+
Number positive	0	5	0	5	0	3

[a] Monkey "Peanuts"; infection sequence D4; day 3 after infection; days of viremia, 3.

[b] Monkey no. H-26; infection sequence D4; day 6 after infection; days of viremia, 3, 4, and 5.

[c] Monkey no. H-32; infection sequence D4; day 10 after infection; days of viremia, 3, 4, and 5.

[d] Symbols: +, One or more plaques per 0.6 ml of explant culture medium in LLC-MK2 plaque assay. 0, No virus recovered.

assay. It is possible that the inhibitors previously demonstrated in this laboratory are responsible in both the human and monkey for virus isolation failures (7). We have successfully recovered dengue virus using the explant technique by using tissues held at refrigerator temperatures for 96 hr postmortem (Marchette and Halstead, *submitted for publication*). This may provide a practical procedure applicable to clinical medicine.

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