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Replication of Dengue and Junin Viruses in Cultured Rabbit and Human Endothelial Cells†

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The flavivirus dengue and the arenavirus Junin are both associated with a hemorrhagic shock syndrome in man. We have demonstrated the replication of these viruses in vitro in both rabbit and human endothelial cells by viral titers and immunofluorescent antibody studies. Rabbit endothelium established in continuous culture was derived from vena cava, while human cells in primary culture were derived from umbilical veins. In rabbit endothelium, dengue-2 virus passaged through monkey kidney monolayer cells (LLC-MK_2) or human lymphoblastoid cells (Raji) produced significantly more virus than the seed obtained from suckling mouse brain (MB). Inoculation of actively dividing, subconfluent human endothelial cells with the LLC-MK_2 dengue virus produced higher viral titers than inoculation of confluent cells. The appearance of Junin virus was delayed beyond that of dengue virus in rabbit endothelial cells although equivalent titers of virus were produced. In human cells, Junin virus was less productive than dengue virus and produced characteristic cycles of virus release. This is the first direct evidence for replication of human hemorrhagic fever viruses in endothelial cells.

Viruses that produce hemorrhagic syndromes, in particular the flavivirus dengue and the arenavirus Junin are a major cause of morbidity and mortality in humans (8, 16). Immunological mechanisms have been considered to play an important role in the dengue shock syndrome (DSS) (8, 13, 20), since this disease generally develops in the presence of dengue viral antigens and circulating anti-dengue antibody (8, 20). However, a recent report has indicated that DSS may develop in primary dengue infection before the demonstration of the early immunoglobulin M anti-dengue antibody (21). DSS may be associated with activation of the complement system by both the classical and alternative pathways (2). Intravascular coagulation has also been observed in these patients and is manifested by thrombocytopenia (2, 8), reduction in coagulation factors, especially factor VIII (7), and plasma fibrinogen (2) with circulating fibrinogen degradation products (2). The mechanisms leading to activation of these effector systems in DSS are poorly understood (8, 20), although circulating immune complexes are considered the most important common denominator (2, 20, 22, 27). In addition, immune complexes are occasionally demonstrated in tissues (3).

Argentinian hemorrhagic fever, which results from the initial exposure to Junin virus (16; M. B. A. Oldstone and C. J. Peters, in *Handbook of Clinical Neurology*, part 1, in press), may be associated with activation of the alternative complement pathway (M. M. E. de Bracco, M. T. Rimoldi, J. I. Maiztegui, P. M. Cossio, and R. M. Arana, Fed. Proc. **36**:1265, 1977). However, neither circulating nor tissue-deposited immune complexes have been demonstrated in these patients (17).

Individuals with either DSS or Argentinian hemorrhagic fever may undergo profound hemorrhagic manifestations (2, 8, 16, 20; Oldstone and Peters, in press), which may not always be entirely attributable to the syndrome of intravascular coagulation (20, 29). On occasion capillary fragility, as evidenced by a positive tourniquet test, may develop without a significant reduction in the platelet count or specific coagulation factors (20, 29). Perhaps these viruses replicate within vascular endothelial cells (11, 18), resulting in either direct or indirect cellular damage. In the present study we have demonstrated in vitro replication of both dengue and Junin viruses in cultured rabbit and human endothelial cells.

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MATERIALS AND METHODS

Viral seeds. Dengue-2 viral seeds used in these experiments were obtained from three sources: (i) mouse brain (MB) seed was derived from the New Guinea C strain at passage 35 in suckling mouse brain (NG-C Sm35); (ii) LLC-MK_2 seed came from a single passage of NG-C Sm35 through a monkey kidney monolayer (LLC-MK_2); and (iii) Raji seed was originally isolated from plasma of a Thai patient (16681 strain), and the virus was passaged through monkey kidney monolayers (BSC/1), a normal Rhesus monkey, adult *Aedes albopictus* mosquitoes, and finally through the B-type lymphoblastoid Raji cell. All dengue-2 viral preparations were passed through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.) prior to use.

Junin virus was an attenuated high passage of the XJ strain (K. Johnson, Center for Disease Control, Atlanta, Ga.).

Rabbit endothelial cells. Cultured rabbit endothelial cells (REVC) were derived from the vena cava of a normal animal (F. Jensen, Scripps Clinic and Research Foundation, and G. Stoner, University of California-San Diego). In essence, the cells were prepared by tryptic digestion of endothelial cells from an isolated perfused rabbit vena cava, followed by cloning to obtain a homogeneous cell population with morphological characteristics of endothelial cells. Analysis of the cultured REVC cells showed that they retained the characteristic intracytoplasmic Weibel-Palade bodies, demonstrated human type B blood group antigen, and formed angiotensin-converting enzyme. In addition, their growth was contact inhibited, and when confluent the cells were unable to be stimulated to divide by addition of fresh serum. Factor VIII production has not as yet been demonstrated in the REVC cells. These properties of endothelial cells have recently been reviewed (9, 15). Cultured REVC cells employed in these experiments were obtained from passages 20 through 45. Cells were maintained in monolayer culture in RPMI 1640 (Flow Laboratories, Rockville, Md.) containing 20% heat-inactivated (56°C for 30 min) fetal calf serum containing glutamine and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml; Grand Island Biological Co., Grand Island, N.Y.). This remained the standard medium for the REVC cells. Suspensions of REVC cells were obtained by trypsin-ethylenediaminetetraacetic acid (Flow Laboratories) digestion of the confluent monolayer, followed by centrifugation (800 × *g* for 5 min) and washing in the standard medium.

Human endothelial cells. Umbilical cord veins were the source of human endothelial cells and were prepared by a described method (9, 15).

Endothelial cells in culture exhibited a characteristic growth pattern in a monolayer, as flat polygonal cells (9, 15) with readily demonstrable Weibel-Palade bodies and intracellular factor VIII, as well as the other characteristic features previously mentioned.

Viral determination. Standard plaque assays were performed for dengue-2 virus using LLC-MK_2 cells (24) and for Junin virus using Vero cells (6).

Viral inoculation of cultured endothelial cells. Subconfluent endothelial cells in 25-cm^2 sterile plastic screw-top flasks (Falcon, Oxnard, Calf.) were inoculated with the various seeds of dengue-2 or Junin virus at an approximate multiplicity of infection (MOI) of 0.05 per cell seeded. After 2 h, the REVC cells were washed three times, and the medium was replaced. The medium was removed at daily intervals, centrifuged, and stored at −70°C for plaque assay; fresh medium was placed on the cells. Viral titers were determined on aliquots of the medium that was changed daily.

After preparation from umbilical cord veins, 2×10^5 human endothelial cells were seeded into 3.5-cm-diameter sterile plastic petri dishes in 3 ml of medium. After varying periods, the cultured cells were inoculated either with the LLC-MK_2 seed of dengue-2 or with Junin virus at an approximate MOI of 0.05.

Demonstration of intracellular viral antigens. Intracellular dengue-2 and Junin viral antigens were demonstrated using an indirect immunofluorescent antibody technique. REVC cells (1.6×10^4) or human endothelial cells (2×10^4) in their respective media were seeded into 1-cm^2 tissue-culture chambers (Lab-Tek Products, Naperville, Ill.). The cells were inoculated 24 h later with LLC-MK_2 dengue-2 or Junin virus at an MOI of 0.05. At varying periods postinoculation, the monolayers were washed and fixed in 95% ethanol. To identify dengue-2 viral antigen, heat-inactivated hyperimmune murine ascitic fluid with a complement-fixing antibody titer of 1:512 was prepared by described methods (4), absorbed with uninfected REVC cells, and added to the monolayer at a dilution of 1:32. After incubation with antiserum at room temperature for 30 min, the monolayer was washed and incubated at 4°C for 30 min with fluorescein isothiocyanate-labeled immunoglobulin G fraction of rabbit anti-mouse immunoglobulin G. Negative controls consisted of endothelial cells incubated in both nonimmune murine ascitic fluid and medium alone, followed by staining with fluorescein-labeled antiserum. Preparations were examined by transmitted light fluorescent microscopy using a Zeiss RA microscope. The method for demonstrating intracellular Junin viral antigens was identical except for the use of antiserum prepared in C57Bl/6J mice after intravenous inoculation with Junin virus.

RESULTS

Viral replication in established culture of rabbit endothelial cells. Dengue-2 viral replication in REVC cells (Fig. 1A) was demonstrated for all viral seeds tested at an MOI of 0.05. However, viral replication was significantly lower with the MB seed. Viral titers were almost identical using the Raji and LLC-MK_2 seeds, with maximal supernatant titers (5.5×10^4 plaque-forming units [PFU] per ml) observed 3 days postinoculation. Dengue-2 viral titers peaked at 2 days when REVC cells were inoculated with the MB seed, although maximum titers (1.1×10^3 PFU/ml) were 50-fold less than with the other two seeds. MB virus was undetected in the supernatant by 4 days, unlike the Raji and LLC-MK_2 seeds, where virus persisted

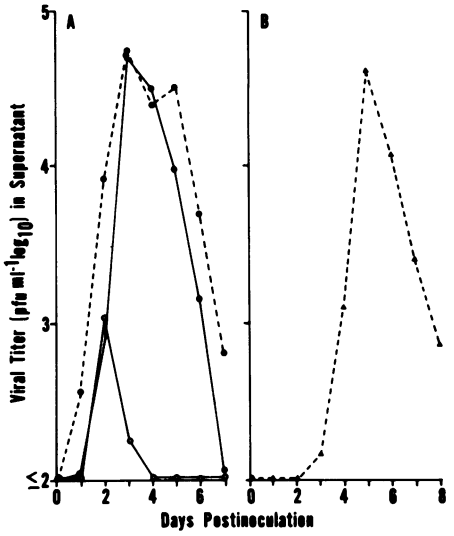

FIG. 1. *(A) Replication of three dengue-2 viral seeds (●——●, Raji; ●----●, LLC-MK$_2$; ○—○, MB seed) in REVC cells inoculated at an MOI of 0.05. (B) Replication of the attenuated vaccine strain of Junin virus in REVC inoculated at an MOI of 0.05.*

for up to 7 days. These observations could suggest the presence of inhibitors or interfering particles in the MB viral seed. In contrast to small, uniform, clearly defined plaques observed with the Raji seed, both the LLC-MK$_2$ and MB viral seeds produced a mixture of both large and small plaques. Using the LLC-MK$_2$ seed (Table 1), we observed that REVC cells produced comparable amounts of intra- and extracellular dengue-2 virus. Virus was still detected in the culture at 8 days.

When REVC cells were inoculated with Junin virus at an MOI of 0.05 (Fig. 1B), supernatant virus was not detected until 3 days postinoculation, with maximal viral titers (4×10^4 PFU/ml) obtained at 5 days. This contrasted with the LLC-MK$_2$ dengue-2 seed, where the virus was first detected after 1 day and reached maximal titers two days before Junin virus.

Viral replication in primary culture of human endothelial cells. Human endothelial cells were inoculated 1 and 5 days after seeding with LLC-MK$_2$ dengue-2 virus at an MOI of 0.05 (Fig. 2A). Confluency had been reached in the 5-day cultures at time of inoculation, whereas the cells seeded at 1 day remained subconfluent. Supernatants of all cultures contained virus 2 days after inoculation. The highest viral titers (4.1×10^5 PFU/ml) were obtained 4 days after inoculating the actively dividing cells which had been seeded for 1 day. Dengue virus was still detected in the supernatant 8 days after inoculation. Endothelial cells which had been seeded 5 and 14 days prior to inoculation with virus attained a maximal titer which was approximately 50-fold less.

TABLE 1. *Intra- and extracellular replication of the LLC-MK$_2$ seed of dengue-2 virus in REVC cells over an 8-day period*

Days postinoculation	Intracellular virus (PFU/dish)	Extracellular virus (PFU/dish)
1	4.2×10^2	2.6×10^3
2	4.7×10^4	9.3×10^4
3	3.0×10^5	2.3×10^5
4	2.0×10^5	7.5×10^4
5	4.4×10^4	2.2×10^4
6	7.2×10^3	4.9×10^3
7	9.4×10^3	1.0×10^4
8	9.9×10^3	2.8×10^4

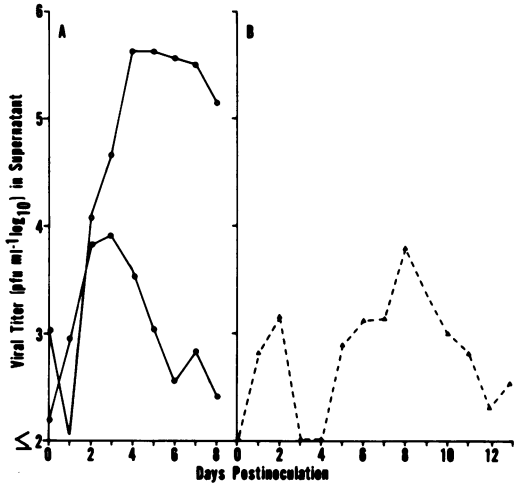

FIG. 2. *(A) Replication of the LLC-MK$_2$ seed of dengue-2 virus (MOI, 0.05) in primary cultures of human endothelial cells inoculated with virus at 1 (○) and 5 (●) days after seeding. (B) Replication of the attentuated vaccine strain of Junin virus in a primary culture of human endothelial cells inoculated (MOI, 0.05) 1 day after seeding.*

Junin virus also replicated in human endothelium cells inoculated 24 h after seeding (Fig. 2B), but demonstrated a consistent cyclical pattern. There was an early viral peak at 1 to 2 days, absence of virus at 3 to 4 days, and further reappearance at 5 days after inoculation. This cyclical variation in viral titers has been observed in arenavirus-infected cell cultures (23; Oldstone and Peters, in press). Maximal titers were observed at 8 days postinoculation, although titers were significantly less than those observed with dengue-2 virus inoculated the same time after seeding.

Intracellular demonstration of viral antigens. Dengue viral antigens were demon-

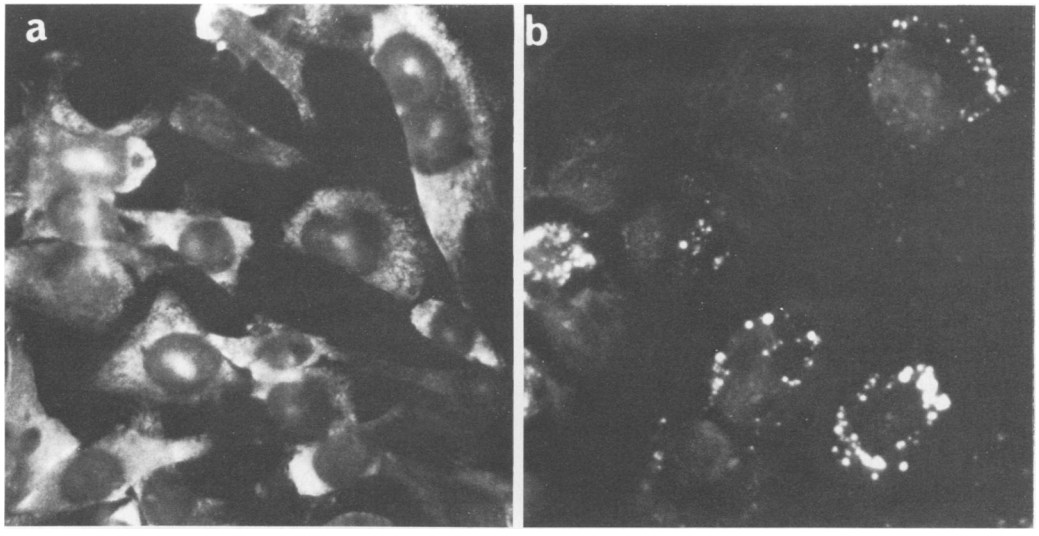

FIG. 3. *Immunofluorescent antibody demonstration of intracellular dengue viral antigens in (a) REVC cells and (b) primary culture of human endothelial cells, inoculated 3 days earlier with the LLC-MK$_2$ seed of dengue-2 virus at an MOI of 0.05 (×480 original magnification).*

strated in both REVC (Fig. 3a) and human (Fig. 3b) endothelial cells 3 days postinoculation using immunofluorescent antibody techniques. In endothelial cells from both species, a maximum of 20 to 30% of the viable cultured cells demonstrated intracytoplasmic dengue viral antigens. Infected cells were most frequently clustered at the actively growing edge of the culture, although sporadic confluent cells also contained viral antigens. Similarly, intracellular Junin virus was demonstrated in only 20 to 30% of cultured rabbit and human endothelial cells, with a similar predominance of infected cells at the periphery of the culture.

Phase-contrast microscopic examination of dengue- and Junin-infected endothelial cells was performed daily. When compared with parallel control uninfected rabbit or human endothelial cells, there was no obvious difference in cytopathic effect observed in the infected cells over the uninfected controls. This demonstrated the apparent non-cytopathogenic nature of these viruses in vitro in cultured endothelial cells.

DISCUSSION

We have demonstrated in vitro replication of dengue and Junin viruses in cultured rabbit and human endothelial cells. Primary cultures of rabbit kidney cells have been unable to replicate dengue virus, although dengue virus has been shown to adsorb to the surface of the rabbit cells, indicating the presence of surface receptors for the virus (J. McCown, unpublished data). Replication in rabbit endothelium suggests that this cell type represents a permissive cell for viral replication in a nonsusceptible host. On the basis of the rabbit endothelial cell data, we extended our study to primary cultures of human endothelial cells, with the subsequent demonstration of both dengue and Junin virus replication.

In addition to endothelial cells, dengue virus replicates in vitro in B-type lymphoblastoid cells (25, 26); in mitogen-stimulated peripheral blood lymphocytes (12, 26); in macrophages (13, 14, 26); and possibly in specifically sensitized lymphocytes (12, 13). Although there are no definite in vivo data to indicate the site(s) of dengue virus replication in humans, the marked lysis observed in lymphoid organs in DSS and the occasional demonstration of viral antigens in lymphoid tissue (1) suggest this is to be a site. The absence of demonstrable intracellular dengue virus in vivo in humans may be related to the focal nature of the infection. In contrast, Junin virus can be readily demonstrated in almost every organ examined (17; Oldstone and Peters, in press).

The work reported here is the first direct demonstration of viral replication in isolated endothelial cells and is the first direct evidence for replication of two important human hemorrhagic fever viruses in vitro in human endothelial cells. Previous data on viral replication in endothelial cells are confined to electron microscopy and fluorescent antibody studies on tissue (5, 10, 18, 19, 28).

Mims (18) and Halstead (11) have suggested

that dengue virus may replicate in vascular endothelium. Evidence of capillary fragility in DSS without the marked reduction in the number of circulating platelets which occasionally occurs argues in favor of damage to vascular endothelium. Our in vitro evidence demonstrating the lack of significant cytopathic effect suggests that direct viral-induced damage does not occur. However, in vivo humoral or cellular effector mechanisms may produce endothelial damage with subsequent loss of vascular integrity. A study of the role of complement, antibody, and cellular effector mechanisms in the lysis of infected cells was not attempted, owing to the small percentage and focal distribution of infected endothelial cells.

Finally, while dengue and Junin virus infections may result in loss of vascular integrity following possible endothelial damage by humoral and cellular effector mechanisms, infected endothelial cells may further contribute to the shock syndrome by other mechanisms, including impairment of their factor VIII production or release of plasminogen activator.

The demonstration of dengue and Junin viral replication in human and rabbit endothelium in vitro does not detract from the suggestion that immune complexes in vivo may be involved in the ultimate immunopathology of the DSS (2, 8, 20), but it offers an additional mechanism to account for the vascular pathology and the resultant hemorrhagic shock syndrome. However, until further studies can be performed with unpassaged strains and comparisons can be made between virulent and avirulent human arenaviruses, these results, although provocative, should not be overinterpreted.

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