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Interaction of Alphaviruses with Human Peripheral Leukocytes: In Vitro Replication of Venezuelan Equine Encephalomyelitis Virus in Monocyte Cultures

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Human peripheral blood leukocytes (PBL) were examined for their ability to support growth of several group A arboviruses in vitro. Cells were refractory to infection with eastern (EEE) and western (WEE) equine encephalitis viruses, whereas Venezuelan equine encephalomyelitis (VEE) virus was shown to infect and replicate to a substantially high titer. When PBL were fractionated into purified subpopulations, only the monocytes were susceptible to predictive VEE virus infection. Lymphocytes treated 24 h before virus inoculation with phytohemagglutinin (10 μg/ml) were capable of propagating significant amounts of VEE virus. A monocytic cell line, J-111, was also susceptible to infection with VEE, EEE, and WEE viruses, whereas a lymphocytic cell line, Raji, was refractory. Additional information on the participation of PBL during human infection with these viruses may add considerably to our understanding of their differing pathogenicities and clinical pictures.

Recent studies have demonstrated that selected viruses differ in their ability to replicate in diverse types of human leukocytes. Virus replication within cultured human monocytes has been demonstrated for vesicular stomatitis (6), 17D yellow fever (21), and measles (13) viruses. Several viruses have been shown to propagate in human peripheral blood lymphocytes (9, 13, 14, 16, 17).

With the exception of dengue viruses and yellow fever vaccine virus, little information is available on the interaction of arboviruses and human peripheral leukocytes. Strain 17D yellow fever virus was shown to replicate and induce interferon in cultures of human leukocytes (21). Studies with nearly homogeneous cultures of each leukocyte type revealed that 17D virus replicated to high titer and induced interferon in human monocytes. A small amount of virus replication took place in unstimulated lymphocytes, whereas phytohemagglutinin-stimulated lymphocytes were capable of supporting virus replication. No virus growth was observed in polymorphonuclear cell (PMN) cultures.

In a series of reports (10-12, 15), dengue viruses were shown to replicate in both monkey and human peripheral leukocyte cultures. In a recent study (20), it was demonstrated that dengue-2 virus replicates in B type cultured human lymphoblastoid cell lines, B lymphocytes, and in macrophages, but not in T type human lymphoblastoid cell lines, T lymphocytes, or PMN.

Since Venezuelan equine encephalomyelitis (VEE) virus shows a predilection for the reticuloendothelial system during human infection (8), a study of this virus' interaction with the peripheral blood leukocytes (PBL) was undertaken. The results reported below indicate that macrophages derived from peripheral blood monocytes support VEE virus growth, whereas unstimulated lymphocytes and PMN cultures do not. Additional experiments with eastern and western equine encephalitis viruses are also reported.

MATERIALS AND METHODS

Viruses. TC-83, the attenuated vaccine strain of VEE virus was used throughout this study and has been described in detail previously (1). The lyophilized vaccine (Merrell-National Labs, Swiftwater, Pa.) was passaged once in duck embryo cell culture in our laboratory. A small plaque mutant of eastern equine encephalitis (EEE) virus, strain Arth 167, was obtained from P. H. Coleman, Center for Disease Control, Atlanta, Ga., as culture fluid from the 13th passage in DEC culture. A further passage was made in duck embryo cell culture just prior to use. The clone 15, B628, attenuated strain of western equine encephalitis (WEE) virus was obtained from Lederle Laboratories (Pearl River, N.Y.) as culture fluid from the second passage in chicken embryo cell culture (19). This preparation was further passaged in duck embryo

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cell culture in our laboratory. The virulent strains Trinidad, PE-6, and B628 of VEE, EEE, and WEE viruses, respectively, were used in several experiments. The identity of all viruses used in the present study was verified by neutralization tests employing specific viral antisera.

Leukocyte preparations. The venous blood of seronegative, nonvaccinated healthy adults was collected in sterile vacuum tubes containing preservative-free heparin (15 U/ml), 0.3 ml for each 10 to 14 ml of blood. To accelerate sedimentation of erythrocytes, 1.5 ml of 5% dextran (molecular weight of 200,000 to 275,000, Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline was added to each 10 ml of heparinized blood. After incubation at 37°C for 30 to 60 min, the plasma, containing leukocytes, platelets, and a small quantity of slowly sedimenting erythrocytes, was aspirated and centrifuged at $225 \times g$ for 10 min to sediment the leukocyte and erythrocytes. The plasma supernatant, now containing principally platelets, was aspirated. The cell pellet was suspended in a large volume of phosphate-buffered saline and centrifuged again. The remaining plasma dextran, heparin, and platelets were drawn off with the supernatant. The washed leukocytes were then suspended in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with unheated fetal calf serum (10%), *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid buffer (25 mM), sodium bicarbonate (1.75 g/liter), penicillin, and streptomycin. The procedures employed in obtaining leukocyte cultures did not alter significantly the cell differential count from that of the donor's whole blood using the morphological criteria outlined by Wintrobe (22). Cell cultures prepared directly from peripheral blood as described here are designated PBL.

Determination of cell viability. Immediately after being placed in culture, the leukocytes were counted in a hemocytometer chamber by use of an erythrocin B vital dye exclusion technique (2, 18). The yield of leukocytes from 50 ml of venous blood averaged 200×10^6 cells; more than 99.5% were viable. Cell concentrations in this study are expressed in terms of viable leukocytes per milliliter of culture medium.

Leukocyte morphology. Samples of leukocyte cultures were prepared for study of cell morphology and for differential counts in the following manner. Samples (1 to 2 ml) of leukocyte cultures were centrifuged at $200 \times g$ for 10 min, the medium was aspirated, and the leukocytes were suspended in 0.1 to 0.2 ml of medium by gentle agitation of the tube. A large drop of the cell suspension was carefully disbursed between two glass cover slips (15 by 15 mm), air dried, and stained with Wright's stain. A total of 500 cells was counted from each culture; at least 100 leukocytes on each of four cover slips were identified as to cell type. The differential counts were reproducible; there was close agreement between each of five sets of 100 cells counted from each culture, and there was no significant variation when the same cover slip was counted on two separate occasions.

Separation of leukocyte fractions. A 10-ml volume of PBL or whole heparinized blood diluted with RPMI-1640 medium was layered onto 4 ml of lymphocyte separation medium (Litton Bionetics, Kensington, Md.) and centrifuged at $400 \times g$ at room temperature for 45 min. The mononuclear cell band and the pelleted PMN fractions were collected and washed three times in 10 ml of RPMI-1640 medium; the final cell concentration was adjusted to 10^7/ml. Adherent monocyte cultures were prepared by seeding six well plastic plates (Linbro Division, Flow Laboratories, Inc., Hamden, Conn.) with approximately 10^7 mononuclear cells. After 1 h of incubation at 37°C, the monolayers were gently washed with RPMI-1640 medium three times to remove the nonadherent cells. The monolayers were removed from the plastic surface with a rubber policeman, and the concentration was adjusted to 10^6 cells per ml.

Virus growth curves and infectivity assay. Cell pellets containing 10^6 cells were suspended in 0.2 ml of virus, resulting in a plaque-forming unit (PFU)-to-cell ratio of 1. The cultures were placed in a stationary position for 60 min at 37°C to allow for virus adsorption. The leukocytes were then washed by three cycles of centrifugation at $225 \times g$ for 10 min; the cell pellets were suspended in fresh culture medium and incubated at 37°C in 15-ml plastic, conical centrifuge tubes (Corning Glass Works, Corning, N.Y.). At timed intervals, the sedimented cells were resuspended by pipetting, and 0.5-ml samples were removed, frozen rapidly, and stored at −70°C. Frozen samples were thawed and titrated by plaque assay which was performed in duck embryo cell culture essentially as described by Dulbecco and Vogt (4). Primary duck embryo cell cultures were prepared from decapitated 13-day-old embryos in a procedure similar to the preparation of chicken embryo cell cultures (4).

RESULTS

VEE virus replication in PBL. An initial experiment was performed to determine the susceptibility of human peripheral leukocytes to VEE virus. Leukocyte cultures from healthy VEE virus nonimmune individuals were prepared and inoculated with 10^6 PFU (0.2 ml) of virus, and samples were harvested at timed intervals for virus assay (Fig. 1). Peak virus growth occurs in these cells within the first 24 h postinoculation, with high titers being maintained throughout the 96-h experimental period.

Virus growth in fractionated leukocyte subpopulations. A series of experiments was next performed to determine which leukocyte subpopulation(s) was responsible for the significant virus growth observed with PBL. Mononuclear cells were separated from PMN by centrifugation of PBL on a discontinuous density gradient. Monocytes were further separated from lymphocytes by their adherence to plastic surfaces. After several washes, the isolated subpopulations were counted, adjusted to 10^6/ml, and inoculated in suspension culture with 10^6 PFU so that growth studies could be performed (Fig. 2). It can be seen that the monocyte cultures were capable of supporting replication of significant amounts of virus, whereas lympho-

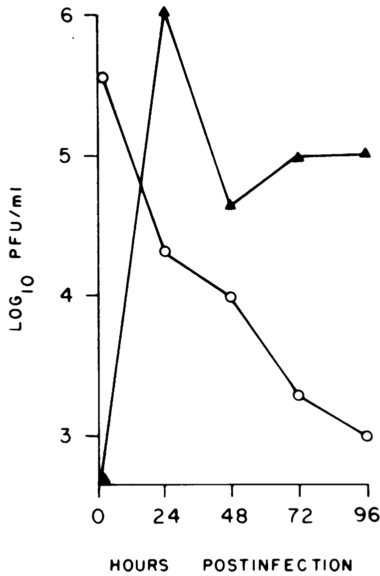

FIG. 1. *Growth of TC-83 virus in human peripheral leukocytes* (▲) *compared to virus stability in cell-free medium* (○).

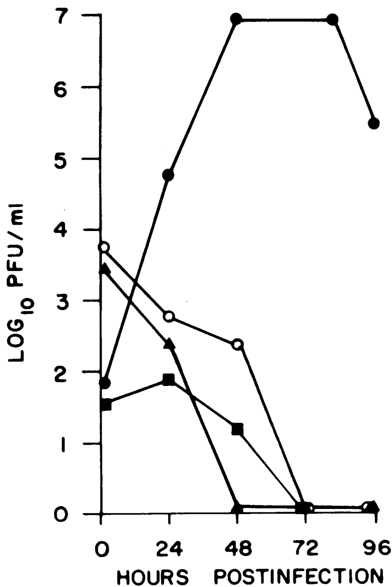

FIG. 2. *Growth of VEE virus in subpopulation of leukocytes. Symbols:* ●, *monocytes;* ■, *lymphocytes;* ▲, *PMNs;* ○, *virus control, no cells.*

cyte and PMN cultures were not. Further evidence of monocyte involvement is illustrated in Fig. 3. The data show the effect of virus infection on the viability of purified cultures of both monocytes and lymphocytes. Infected and noninfected control cultures of each cell type were examined daily for viable cells as described in Materials and Methods. Infected monocyte cultures showed a precipitous decrease in cell numbers compared to uninfected control over a 4-day period. Lymphocytes, whether infected or not, demonstrated no significant drop in cell numbers over the same time period.

Effect of PHA on virus growth in lymphocytes. Several reports (5, 7, 16) have described the enhanced virus growth in lymphocyte cultures by the nonspecific mitogen, phytohemagglutinin (PHA). Since unstimulated lymphocytes did not significantly allow propagation of VEE virus in the present study, we examined virus growth in PHA-stimulated cells. Purified cultures of peripheral lymphocytes were treated with 10 μg of PHA (Difco Laboratories, Detroit, Mich.) per ml 24 h before virus inoculation. Cells were washed, enumerated, inoculated with 10^6 PFU of VEE virus, and sampled daily for virus growth; control cells not receiving PHA were run concurrently. As shown in Fig. 4, unstimulated lymphocytes did not support replication of VEE virus. In contrast, PHA-stimulated cells were highly susceptible, with virus production reaching significantly high titer.

Resistance of human leukocytes to EEE and WEE viruses. Two related group A arboviruses, EEE and WEE, were examined for their ability to replicate in human peripheral mononuclear cells. Replicate cultures of cells were inoculated with varying concentrations of EEE and WEE viruses under conditions described previously for VEE virus. A positive control for virus growth employing TC-83 virus was run in parallel. Comparative growth studies of these viruses can be seen in Table 1. TC-83 virus replicated at all doses tested with titers corre-

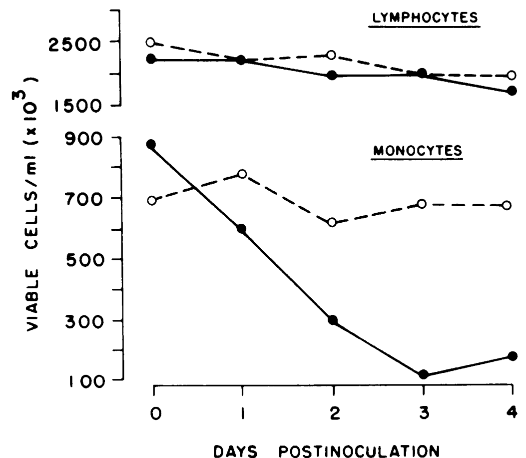

FIG. 3. *Effect of TC-83 virus infection on viable cells in purified populations of monocytes and lymphocytes. Symbols:* ●, *infected;* ○, *uninfected controls.*

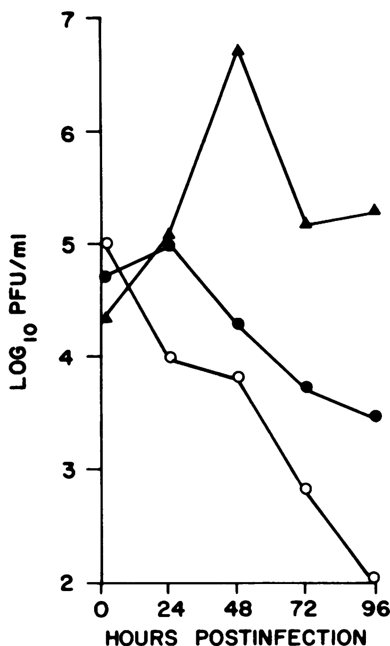

FIG. 4. *Effect of PHA treatment on the growth of TC-83 virus in purified population of lymphocytes. Symbols: ▲, lymphocytes treated with PHA (10 μg/ml); ●, lymphocytes untreated; ○, virus control, no cells.*

sponding directly to increasing virus input. No detectable growth of EEE or WEE viruses was observed with any of the inocula tested in this or subsequent experiments (data not shown). The virulent virus strains showed growth characteristics similar to that shown for the avirulent strains of each of the three arboviruses tested.

Arbovirus growth in leukocyte cell lines. Two human cell lines, J-111 (ATCC, Rockville, Md.) from monocytic leukemia and Raji (ATCC) from Burkitt lymphoma, were tested for their ability to propagate several group A arboviruses. Cells were inoculated with virus as described previously for normal peripheral leukocytes and observed for virus growth. Results of this comparative study are found in Table 2. The VEE vaccine strain, TC-83, its virulent parent, Trinidad, and both EEE and WEE viruses all grew to exceptionally high titer in J-111 cells. No significant growth of any virus could be detected in the lymphoid cell line (Raji). These results appear to confirm our previous observation that VEE virus propagates in monocytes but not appreciably in cells of lymphocyte origin. It is of interest to note that, although EEE and WEE viruses did not grow in normal peripheral monocytes, they did grow in the monocytic cell line (J-111).

DISCUSSION

VEE virus has been demonstrated to replicate to moderately high titers in human peripheral leukocytes. Peak titers were observed in PBL 24 h after inoculation, with the growth curves resembling that of other VEE virus-susceptible cells.

When PBL were fractionated into purified macrophage (monocyte), lymphocyte, and PMN cultures, it was seen that only the macrophage culture was capable of producing significant virus yields. Although lymphocyte cultures were incapable of supporting growth of VEE virus under normal culture conditions, virus growth was observed in these cultures when cells were treated with PHA 24 h before virus inoculation. This finding, although quite intriguing, is not unusual since it has been described previously for several other animal viruses. (3, 5, 7, 16).

Concomitant with virus growth, monocyte cell

TABLE 1. *Comparison of group A arbovirus replication in human PBL*

Virus	Virus inoculum (PFU/ml)	Virus growth ($\log_{10}$ PFU/ml) at day:		
		0	1	2
EEE				
Arth 167 strain (avirulent)	10^3	0	0	0
	10^4	2	0	0
	10^6	3	2	1
PE-6 strain (virulent)	10^6	3	1	0
WEE				
Clone 15 strain (avirulent)	10^3	0	0	0
	10^4	2	0	0
	10^6	3	2	1
B628 strain (virulent)	10^6	2	1	0
VEE				
TC-83 strain (avirulent)	10^3	0	2	5
	10^4	1	3	6
	10^6	3	5	6
Trinidad strain (virulent)	10^6	2	3	4

TABLE 2. *Growth of arboviruses in leukocyte cell lines*

Virus (10^6 PFU/10^6 cells)	Virus growth[a] (PFU/ml)	
	J-111	Raji
TC-83	1×10^9	2×10^3
Trinidad	1×10^8	5×10^2
EEE	5×10^8	1×10^3
WEE	1×10^9	7×10^2

[a] At 48 h postinoculation; assayed on duck embryo cell monolayer cultures.

numbers dropped significantly in infected cultures whereas control, noninfected cell counts remained relatively constant throughout the experiment. No significant difference was noted in cell numbers between control and virus-inoculated lymphocyte cultures. These results tend to support the premise that the monocyte is the VEE virus-susceptible cell and, in addition, that the infection of these cells is cytolytic in nature.

The finding that EEE and WEE viruses, unlike VEE virus, did not propagate in human peripheral leukocytes may possibly explain some of the observed differences in pathogenicities for these viruses. Limited observations in humans and certain animal species (8) demonstrated VEE infection at times to be pantropic, with demonstrable effects on the reticuloendothelial system than that shown for either EEE or WEE viruses. It is interesting to speculate that, in vivo, VEE virus-infected monocytes may spread the infection to other tissues or organs of the reticuloendothelial system, thus resulting in a fulminant, more generalized infection. Although most of the experiments in this study employed modified attentuated viruses, similar virus growth and host cell susceptibility were obtained with virulent unattenuated virus strains (Table 1). Studies examining the role of monocytes during VEE virus infection in both human vaccinees and animal models, are currently in progress.

Experiments employing two leukocytic cell lines and their interaction with VEE, EEE, and WEE viruses seem to confirm the observation that the monocyte is the virus-susceptible cell. Raji, the B lymphocyte line, was refractory to all viruses tested. In contrast, all three arboviruses employed in this study grew prolifically in the monocyte line (J-111). It is of interest to note that this malignant monocyte line was capable of supporting growth of EEE and WEE viruses, whereas normal peripheral monocytes were not.

The present study demonstrates the in vitro susceptibility of peripheral human monocytes to VEE virus and their potential role in the pathogenesis of this virus in human disease. Studies on the interaction of VEE virus and leukocytes from immune individuals and the identification of the monocyte binding site for this virus are in progress.

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