JOURNAL OF VIROLOGY, Aug. 1968, p. 787-792

Vol. 2, No. 8
Printed in U.S.A.

Vaccinia Virus Replication and Cytopathic Effect in Cultures of Phytohemagglutinin-treated Human Peripheral Blood Leukocytes

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Received for publication 3 June 1968

Titers of vaccinia virus consistently increased in cultures of washed phytohemagglutinin-treated, peripheral blood leukocytes of a vaccinated adult. Concomitantly, a gradual rise occurred in the numbers of infected leukocytes, as determined by the infective center assay. Increase in viral titer was accompanied by cell injury, decline in cell numbers, and decreased acid production. Leukocytes not pretreated with phytohemagglutinin appeared to form infective centers after exposure to the vaccinia agent, but they did not replicate infectious virus. For viral replication, the continuous presence of phytohemagglutinin was required.

The present investigation was designed primarily to define parameters of a cell culture system in which quantitatively reproducible results can be obtained in analyses of factors that influence the capacity of leukocytes to support viral replication. The system chosen consisted of human blood leukocytes and vaccinia virus, an agent that has not yet been reported to multiply in such cells, although it was the first to be cultivated in the white blood cells of lower animals (3).

MATERIALS AND METHODS

Viruses. Two lines of the Massachusetts Antitoxin Laboratory strain of vaccinia virus were used. The first, chick cell vaccinia virus (CCVV), had been carried through many passages in embryonated eggs and was partially purified by differential centrifugation. The second line of virus, human cell vaccinia virus (HCVV), was derived from calf lymph smallpox vaccine and was passed several times in monolayer cultures of strains of cells developed from human placental tissue by Elizabeth A. Grogan. Titers of stock CCVV and HCVV were 6×10^7 and 3×10^6 plaque-forming units per ml (PFU/ml), respectively, when titrated in cultures of the AH-1 line (5) of grivet monkey renal cells (GMK). CCVV and HCVV were both employed because at first it was postulated that a human cell-adapted virus might replicate to a greater extent in human leukocytes. However, clearly defined differences in this respect were not observed.

White blood cell cultures. Leukocytes from a single healthy vaccinated adult were employed throughout. Erythrocytes from blood taken in a syringe wet with heparin (approximately 20 to 50 units/ml) were sedimented either by gravity or by addition of a 1% solution of dextran, and the leukocyte-rich plasma was removed. Leukocytes were washed three times and resuspended in Eagle's medium containing twice the usual concentration of amino acids and vitamins and 15% fetal bovine serum. The initial concentration of white cells, which were often mixed with some erythrocytes, was adjusted to 6×10^5 white cells per ml. Cultures were maintained at 37 C in an atmosphere of 5% CO_2 in air. Where indicated, phytohemagglutinin-M; (PHA Difco) diluted to a final concentration of 200 μg/ml was added to the culture.

Inoculation of virus. After 3 days of incubation at 37 C, nearly all of the cells were found suspended in the culture fluid. Before inoculation, the medium was separated by centrifugation and was replaced. As routine, after 2 to 5 hr in a roller wheel at 37 C, the cultures were washed repeatedly with neutralized Hanks' solution, and a medium containing PHA at the original concentration was replaced.

Viral titrations. A standard plaque assay was used for all titrations. Total virus was measured by titrating the entire contents of cultures which were previously frozen and thawed rapidly three times. Each titration was performed on two or more AH-1 GMK monolayer cultures in petri dishes. After 2 hr of incubation at 37 C, the inoculum was removed, and an overlay consisting of Eagle's basal medium with 2% fetal calf serum and 1.5% agar was added. After 3 days at 37 C, the preparations were stained with neutral red, and the plaques were counted.

Assay of infective centers. Most of the virus free in the medium was removed by repeated washing of the cells (usually six times). To neutralize virus adherent to the cell surface, the cells were then treated with hyperimmune goat antivaccinia serum diluted 1:4

(virus neutralizing titer >1:1,000). As a control, preimmunization goat serum was employed. (These reagents were a gift from Philip Coleman, National Communicable Disease Center, Atlanta, Ga.). After the cell-serum mixtures were incubated at 37 C for 1 to 2 hr, the cells were counted. Dilutions of the mixtures were prepared, and samples of each dilution were distributed on duplicate GMK monolayer cultures, which were then handled as described in the preceding section. Portions of each dilution were also centrifuged at 1,500 rev/min for 5 min, and the infectivity titers of supernatant fluids were determined.

Mitotic indices and cytomorphology. The proportion of cells in mitosis was determined by the method of Moorehead (6). Cellular morphology was examined in air-dried coverslip smears of cells, fixed in methyl alcohol, and Giemsa stained.

RESULTS

Demonstration of viral replication. In preliminary experiments, PHA-stimulated leukocyte cultures were exposed to decreasing input multiplicities of both CCVV and HCVV. Portions of the leukocyte cultures were assayed for total virus content immediately after washing and 3 days after incubation. A 3 to 80-fold increase in infectivity titer with both strains occurred during this interval. The increase, however, was greater in cultures that were infected with less than 1 PFU per cell.

To establish that the increase in viral titer was the result of replication in infected cells, and not due to other factors such as disaggregation of clumped viral particles, infective centers were determined on the day of inoculation and again 3 days later. The results (Table 1) demonstrated an increase in infective centers, not neutralizable by excess antiserum, from approximately 0.4% of cells on the day of inoculation to about 18% of cells on the third day after infection. In the cultures treated with normal serum, there was an increase in the proportion of plaque-forming cells from 5% on the day of inoculation to 60% 3 days later. That these increases were associated with an increase in total virus content is also apparent from the data (Table 1). The observed increment in viral titers in the antiserum-treated cultures presumably reflects an increase in intracellular virus.

Determination of the kinetics of viral replication demonstrated gradual increases in total virus, extracellular virus, and antiserum-treated infective centers after inoculation of PHA-treated leukocytes. Total virus did not increase until more than 8 hr following infection. Maximal levels of virus were reached approximately 72 hr after infection with an inoculum of 0.25 PFU per cell. At the peak of viral increase, extracellular virus comprised less than one quarter of the total virus in the culture. Assuming that the increase in total virus observed during the first 20 hr of infection represents replication during a single cycle in the cells initially infected, the yield per infected cell was calculated as 30 or 40 PFU on the basis of data obtained in two experiments.

Effect of vaccinia on cell numbers and morphology of leukocytes. Figure 1 graphically summarizes the results of an experiment in which the number of cells in cultures treated with stock virus, ultraviolet-irradiated virus, or suspending medium

TABLE 1. *Infective centers and cell-associated virus after exposure of PHA-treated leukocytes to vaccinia virus*[a]

Time (day)	Tube	Treatment	Cells associated with virus (%)	No. of cells[b] in culture	Calculated no. of cells associated with virus	Observed PFU/ml after freezing and thawing
0	1	Antiserum	0.3	50,000	150	105
0	2	Antiserum	0.5	60,000	300	100
0	3	Normal Serum	5.3	72,000	3,800	1,420
0	4	Normal Serum	4.5	98,000	4,400	1,920
3	5	Antiserum	11	75,000	8,300	5,000
3	6	Antiserum	24	35,000	8,400	23,000
3	7	Normal Serum	63	25,000	15,700	100,000
3	8	Normal Serum	54	25,000	13,500	66,000

[a] PHA-treated leukocyte cultures, after 3 days incubation at 37 C, were exposed to 1 PFU/cell of HCVV. After 4 hr at 37 C, cells were washed six times. Portions of cells then treated with either goat antivaccinia serum or normal goat serum were incubated for 1 hr, and infective centers were determined. Remaining cells were washed three times to remove serum, resuspended, counted, frozen and thawed three times, and assayed for total virus content. The entire procedure beginning with the washes was repeated 3 days after exposure.

[b] Cell counts made after washing to remove serum, immediately before cultures were frozen.

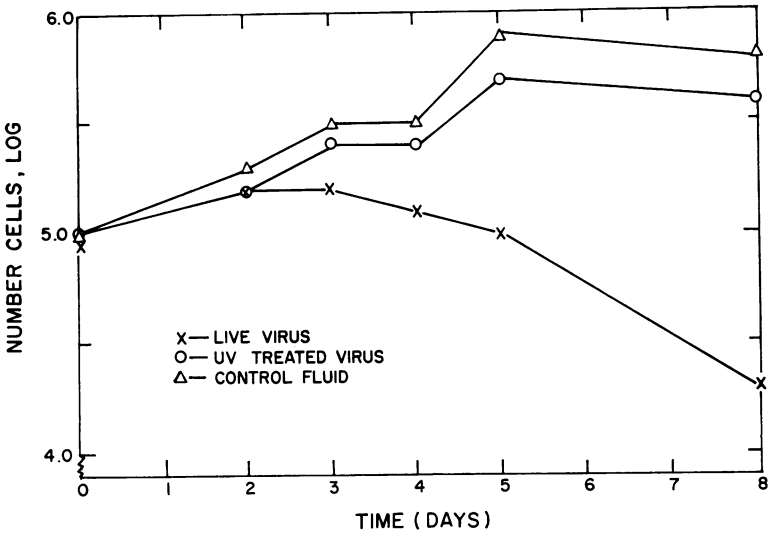

FIG. 1. *Effect of vaccinia virus on numbers of cells in PHA-treated leukocyte cultures. PHA-treated leukocyte cultures after 3 days of incubation at 37 C were inoculated with either HCVV at a multiplicity of 0.25 PFU/cell, or with the same material previously irradiated with ultraviolet light for 30 min (infectivity titer 0.006 of the stock virus), or with suspending fluid. After 2 hr at 37 C, cells were washed six times, resuspended in growth medium, and reincubated. Portions from each of the cultures were removed at the indicated times, and intact cells were counted. Virus added at zero time.*

for virus was determined at intervals throughout a period of 8 days. It is evident that the leukocytic populations exposed to control fluid or to irradiated virus, increased significantly (8- and 5-fold, respectively). In contrast, in the culture exposed to active vaccinia virus, cell numbers at first increased slightly and then steadily diminished.

In an experiment similar to that just described, stained films of leukocytes infected with virus or treated with control fluid were examined on successive days for changes in cell morphology. The most striking alterations, first observed about 2 days after infection, consisted either in condensation of the chromatin or fragmentation of the nucleus. Cells thus affected (*see* Fig. 2) appeared thereafter in increasing numbers in the infected cultures. In one preparation, cells exhibiting such changes composed about 15 to 20% of 1,000 cells counted on the fourth day after inoculation. Degenerating cells exhibiting comparable features were rarely observed in control cultures at this time.

Effect of phytohemagglutinin. Nahmias and his co-workers originally reported (7) that multiplication of herpes simplex virus in leukocytes did not proceed in the absence of PHA. In these cells, enhancement of replication by PHA has subsequently been observed by other investigators with vesicular stomatitis and mumps viruses (1, 2). To determine whether replication of vaccinia virus in these cells was likewise dependent on this reagent, leukocyte cultures were established with decreasing concentrations of PHA (Table 2). Viral increase was recorded in cultures treated with 200 μg/ml and 50 μg/ml of PHA, respectively. With smaller amounts of PHA and in its absence, no increase in virus was observed. Since cell counts of control uninfected cultures demonstrated no major differences, the effect of PHA on viral replication could not be attributed to variation in the number of cells. It is of interest (Table 2) that the mitogenic effect of PHA was evident in amounts which failed to enhance viral replication.

Experiments designed to explore the mode of action of PHA suggested that virus enters both PHA-stimulated and unstimulated cells, but it does not replicate in the absence of PHA. Leukocytes were cultivated for 3 days in the presence or absence of PHA and were later exposed to virus. The proportion of infective centers was then determined (Table 3). Infective centers formed both in the absence and in the presence of PHA. Their incidence, however, was from two to four times greater in PHA-treated cultures. Another experiment showed that, in contrast to PHA-stimulated cells, the numbers of infective centers in cultures not treated with PHA diminished with time, along with the amount of virus present in the whole culture.

It was further determined that viral replication

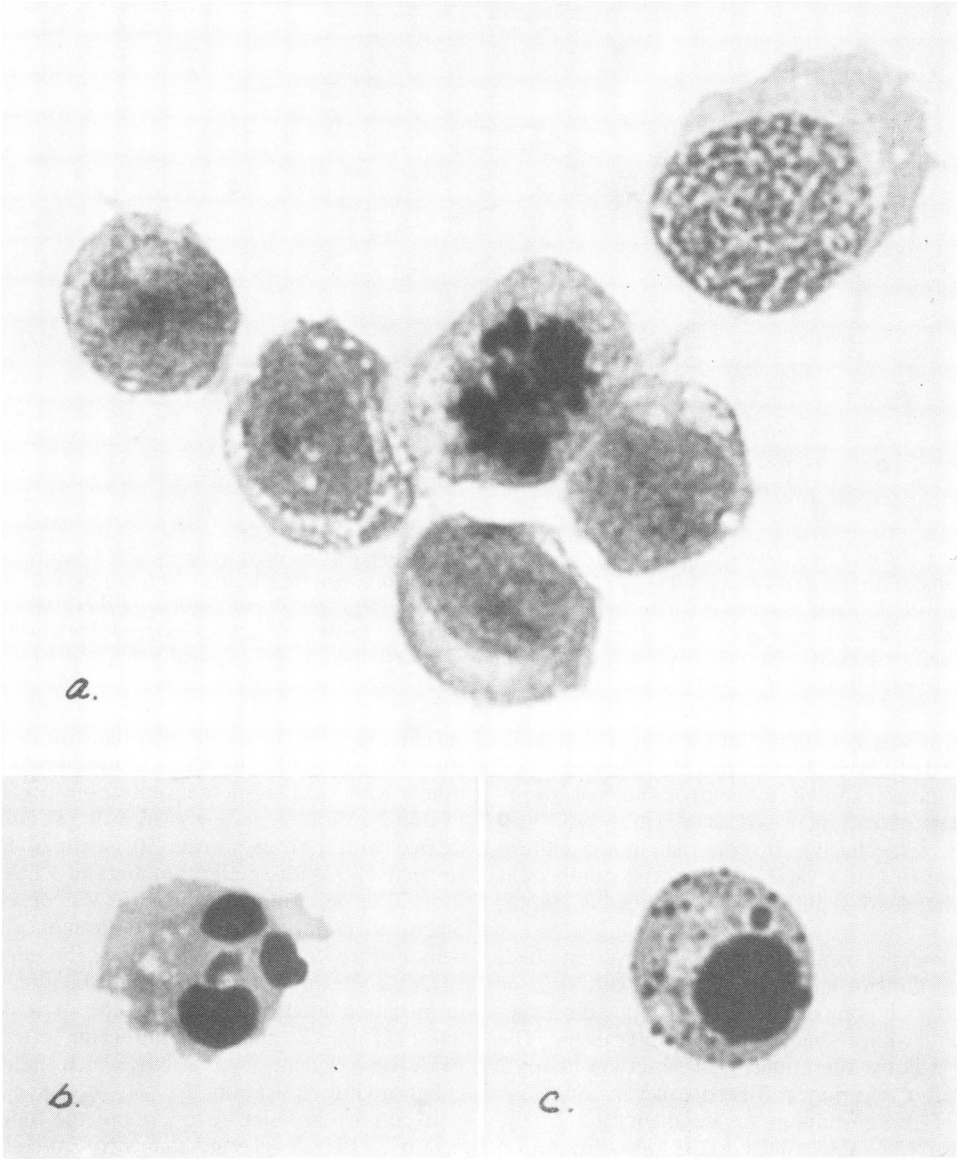

FIG. 2. *Cytopathic effect of vaccinia virus in leukocyte cultures. Cells after 7 days total incubation and 4 days after exposure either to HCVV, 0.25 PFU/cell, or virus suspending fluid. Oil immersion, × 2,000. (a) Exposed to control fluid: a group of "blastoid" cells including a mitotic figure. (b and c) Single representative cells from culture exposed to HCVV. Note early karyorhexis in (b) and pyknotic nucleus with heavy cytoplasmic granulation in (c).*

depended on the continued presence of PHA. When PHA was replaced immediately after infection of PHA-stimulated cells, viral replication proceeded at the usual rate (Table 4). When PHA was withdrawn and replaced after an interval of 46 hr, replication was delayed. When PHA was not replaced at this time, no significant replication occurred subsequently. In this experiment, more than 75% of leukocytes in uninoculated cultures which had been continuously exposed to PHA were found to present a "blastoid" appearance, whereas the majority of cells in cultures from which PHA was withdrawn were regarded as "lymphocytoid" by 88 hr.

DISCUSSION

The application of a standardized procedure for the cultivation of vaccinia virus in human

TABLE 2. *Failure of vaccinia virus to replicate in leukocyte cultures in the absence of PHA*[a]

Dilution of PHA[b]	Inoculated cultures			Uninoculated cultures[c]		
	Log PFU/ml		Virus increase	Cells/ml	Lymphoblastic response[d]	Mitosis[e] (%)
	Day 0	Day 3				
Undiluted	2.5	>3.9	>25-fold	3.5×10^5	++++	4.1
1:4	2.6	>3.9	>20-fold	3.1×10^5	++++	4.6
1:16	2.5	2.5	None	2.4×10^5	+++	5.9
1:64	2.2	2.0	None	2.8×10^5	++	2.4
None	2.3	2.1	None	1.6×10^5	+	0.4

[a] Leukocyte cultures which had been treated with dilutions of PHA or with no PHA and incubated for 3 days at 37 C were exposed to 0.01 PFU/cell of CCVV. After 5 hr at 37 C, cells were washed, resuspended in growth medium containing PHA at the original dilution or no PHA, and again placed at 37 C. Total virus was assayed on the day of inoculation and 3 days later.

[b] PHA, phytohemagglutinin M, originally reconstituted in growth medium at a concentration of 200 μg/ml and diluted as shown.

[c] Total cell counts, estimates of lymphoblasts, and mitotic counts made in control cultures of 6 days in vitro age.

[d] Lymphoblastic response: (estimates) ++++, >75%; +++, 50–75%; ++, 25–49%; +, <25%.

[e] Per cent mitotic figures based on metaphases counted in 1,000 cells in replicate cultures 4 hr after addition of colchicine.

TABLE 3. *Formation of infective centers after exposure to vaccinia virus of leukocytes treated and not treated with PHA*[a]

PHA treated			Not PHA treated		
No. of cells	No. of plaques	(%)	No. of cells	No. of plaques	(%)
28,000	33	0.15	24,000	10	0.04
9,500	33	0.35	8,000	7	0.09
3,167	16	0.52	2,666	6	0.23

[a] Two sets of leukocyte cultures, one established with PHA at a concentration of 200 μg/ml, the other established without PHA, were incubated for 3 days at 37 C and then exposed to 0.4 PFU/cell of HCVV. After 3 hr at 37 C, the cultures were washed six times and then incubated for 1 hr with antiserum. Cells were counted, diluted, and plated on AH-GMK monolayers.

TABLE 4. *Effect of withdrawal of PHA during varying intervals on the replication of vaccinia virus in leukocyte cultures*[a]

Time after inoculation	Logarithm of total virus (PFU/ml)		
	PHA replaced at 0 hr	PHA replaced at 46th hr	PHA not replaced
hr			
0	3.1	3.0	3.0
19	3.0	3.0	2.9
46	4.5	3.3	3.3
69	5.4	4.3	3.5
88	>5.6	>5.6	3.6

[a] PHA-treated leukocyte cultures, after 3 days of incubation at 37C, were exposed to 0.25 PFU/cell of HCVV. After 3 hr at 37 C, cells were washed six times, resuspended in medium, and divided into three portions. PHA (200 μg/ml) was replaced immediately or after 46 hr or not at all. Specimens were removed at times shown from each sample and assayed for total virus.

peripheral blood leukocytes clarified several features of the replication of this agent in these cells. Results of experiments on the formation of infective centers indicate that the virus may become associated with many of the cells and in a significant proportion (more than 25% in most experiments) assume an intracellular position. When taken with determinations of the total amount of virus present in cultures at various intervals after infection, these results imply that infection spreads among the cell population. Finally, they allow an estimation of the yield of PFU per leukocyte which is of the same order of magnitude as that found for cells derived from fixed tissues (4).

These studies revealed a progressive morphological alteration in the leukocytes after exposure in vitro to vaccinia virus. These cytopathic changes were infrequent during the first 2 days following addition of the virus and thus are probably to be distinguished from the toxic effect of the virus reported in an earlier study (8). Although changes comparable to those we observed may occasionally be encountered in uninfected cultures, they are clearly more numerous in infected systems.

The cells thus affected appear to be the PHA-

induced lymphoblasts that predominate in cultures 3 days after their establishment. The possible contribution of blood macrophages that might have been present to the observed viral replication requires further study. However, too few macrophages are present to account for the number of infective centers measured. It seems probable that granulocytes do not play a major role in viral replication since most are degenerated at the time the cultures are inoculated. We recently found that nearly homogeneous suspensions of lymphocytes prepared by filtration of mixed leukocytes through a column of glass beads (9) were capable of supporting viral replication after the addition of PHA.

The data presented in this paper favor the hypothesis that PHA induces intracellular changes in the mature lymphocyte which are essential for viral multiplication rather than adsorption and penetration, since cells can become infected in the absence of this reagent (Table 3), and since viral replication in infected cells does not follow unless PHA is constantly present (Table 4).

It seems likely that enhancement of viral growth is related to the "blastogenic" and "mitogenic" effects of PHA rather than to another unrecognized activity of this chemically ill-defined substance. The data presented do not allow a choice between these alternatives. When purified materials become available, it may be possible to choose between them, as well as to resolve the paradox presented by the viral enhancing effect of PHA and its capacity to stimulate interferon production (10).

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-01992-11-VR and Postdoctoral fellowship 5-F2-AI-31,453-02 from the National Institute of Allergy and Infectious Diseases.

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