Replication of Poliovirus in Phytohemagglutinin-stimulated Human Lymphocytes

FRANK T. C. WILLEMS,¹ JOSEPH L. MELNICK, AND WILLIAM E. RAWLS

Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas 77025

Received for publication 20 January 1969

Poliovirus replication has been studied in human lymphocytes during the course of blastogenesis under phytohemagglutinin (PHA) stimulation. Enhancement of virus replication in PHA-stimulated leukocyte cultures was due to an increase in number of virus-producing cells. Virus yield was approximately 10 plaque-forming units (PFU) per producing cell, both in stimulated and in nonstimulated cultures. Adsorption and penetration studies showed that freshly drawn lymphocytes (unlike other leukocytes) were resistant to virus infection, but they became susceptible to the virus during PHA stimulation. Also, the eclipse of the virus after penetration was enhanced during blastogenesis of the lymphocytes. Our findings suggested that the monocytes in the leukocyte cultures were infected initially. In PHA-stimulated cultures, the virus then spread to lymphocytes which became susceptible to virus infection during blastogenesis. Polymorphonuclear cells died within 24 to 48 hr after initiation of the cultures and apparently could not support poliovirus replication.

Poliovirus replicates *in vitro* in human leukocytes cultured without phytohemagglutinin (PHA) stimulation, but the virus infects only a small proportion of the cells in these cultures. Since the virus does not replicate in either purified fresh lymphocytes or polymorphonuclear leukocytes, it was suggested that monocytes were the host cell for the *in vitro* replication of the virus (10). Similar observations have been made with vesicular stomatitis virus (VSV) in human leukocytes (8). However, purified lymphocytes which were stimulated with PHA supported the replication of VSV (7).

We have previously demonstrated that poliovirus replicates in PHA-stimulated leukocyte cultures in which the blastogenic response of the lymphocytes is partially inhibited by the virus (27a). Our studies were undertaken to determine whether poliovirus replication was enhanced in PHA-stimulated leukocyte cultures and to follow the infectious process of the virus in the presence and absence of PHA stimulation.

MATERIALS AND METHODS

Virus and virus assay. Poliovirus type 1 (Mahoney strain) stocks were prepared in primary green monkey kidney cells, and the virus was assayed in BSC-1 cells

by the plaque counting method (27a). The virus stocks were tested and found to be free of mycoplasma.

Neutralization test and antisera. Antibodies to poliovirus were assayed by the plaque reduction method. Equal volumes of diluted sera and 200 plaque-forming units (PFU) of virus were mixed and incubated at 37 C for 1 hr. The mixture was then assayed for surviving virus, and the dilution of antiserum which produced a 90% reduction of PFU was taken as the antibody titer. Equine antiserum which had a neutralizing titer of 1:9,000 was used where indicated. Sera from all leukocyte donors contained antibodies to poliovirus type 1. All antisera were inactivated by heating at 56 C for 30 min.

Leukocyte cultures. Leukocyte cultures were prepared as described elsewhere (27a). To remove antibodies for poliovirus type 1, the cells were washed three times with Hanks' basal salt solution (BSS). To test for residual antibody, suspensions containing 8×10^6 washed cells/ml were frozen and thawed twice and treated with 1,000 PFU of poliovirus contained in 0.1 ml. There proved to be no significant neutralization of poliovirus by ruptured cells or by supernatant fluids from cell suspensions after the last wash. Cultures contained approximately 2×10^6 cells in 2 ml of medium. PHA M (Difco, lot no. 510957) was diluted 1:50 in saline, and 0.1 ml of this dilution was added to each culture. Since the lot of fetal calf serum used in the leukocyte culture medium may influence the PHA response of lymphocytes (17), a single lot of fetal calf serum was used throughout.

¹ On leave from the University of Nijmegen, The Netherlands.

Macrophages. Macrophages were obtained from patients undergoing peritoneal dialysis. Fluid, collected after dialysis, was pooled, and the cells were sedimented by centrifugation at 1,200 rev/min for 10 min. Cells were washed twice with Hanks' BSS, counted in a hemocytometer, and tested for viability by the dye exclusion method with trypan blue (21). Cultures were prepared in the same way as described for leukocyte cultures, and contained 2×10^6 cells per 2 ml of medium. Differential counts of the cells revealed that at least 70 to 80% were macrophages, 12 to 20% lymphocytes, and 4 to 10% polymorphonuclear cells.

Infectious center assay. Infected cells were assayed according to the method described by Gresser and Chany (10). Samples of infected leukocytes were removed and washed once with Hanks' BSS. Cells were resuspended in 1 ml of a 1:100 dilution of equine polio antiserum or 1:10 dilution of human polio antiserum and incubated for 30 min at 37 C. Excess antibody was removed with two washes of 10 ml of Hanks' BSS, and the cells were diluted in 10-fold increments. Of each dilution, 0.2 ml was inoculated onto monolayers of BSC-1 cells. After a 2-hr adsorption period, the monolayers were carefully overlaid with agar, and plaques were counted after 2 to 3 days of incubation. The number of leukocytes in each sample was counted in a hemocytometer, and viability of the cells was assayed. Plates of BSC-1 cells were infected with poliovirus at a multiplicity of 4 PFU/cell. After a 30-min adsorption period, the cells were dispersed by trypsinization and plated for infectious centers as above. Assuming that all cells were infected, the efficiency of the assay procedure ranged from 43 to 64% with a mean of 52%.

RESULTS

Preliminary experiments were performed to determine the effect of different donors on poliovirus yield in leukocyte cultures. Cultures containing 10^6 cells were prepared from four donors. Cultures were infected with poliovirus, stimulated with PHA, and incubated for 6 days, and then the virus produced was assayed. A reproducible difference was noted among the donors, but there was no relationship between the final virus yield and the number of surviving "blast" cells from each donor. For this reason, leukocytes from one donor whose cells showed the highest yield of virus at 6 days were used for the experiments described below.

Poliovirus replication in leukocyte cultures in the presence or absence of PHA stimulation. Four groups of leukocyte cultures were prepared and inoculated with virus. After a 2-hr adsorption period at 37 C, all cultures were washed 3 times with 10 ml of Hanks' BSS, and 2 ml of medium was added. Group A received PHA and was infected with virus at a multiplicity of 5 PFU/cell. Group B also received PHA, but was infected with virus at a multiplicity of 0.005 PFU/cell. Groups C and D received the same high and low

input of virus but no PHA. Four samples of each group were taken initially, and four samples at daily intervals for 10 days. Duplicate samples were used to determine total virus yield and to determine the proportion of cells producing virus by infectious-center assay.

Poliovirus replicated in both PHA-stimulated and nonstimulated leukocyte cultures (Fig. 1). There was a greater virus yield in stimulated cultures, and this could be accounted for by an increase in the number of cells actively producing virus. The virus yield per cell in all four groups of cultures was approximately 10 PFU per cell per 24 hr. A maximum of 10% of the cells were found to be producing virus; this maximum occurred on the 3rd to 4th day in cultures of leukocytes receiving 5 PFU/cell plus PHA. The fraction of cells producing virus and the total virus yield per culture declined after the 5th day. The highest proportion of infected cells and the greatest virus yield occurred later in those cultures receiving less virus and in nonstimulated cultures.

The effect of PHA stimulation on the susceptibility of the leukocytes to virus infection was examined more closely. Leukocytes were stimulated with PHA, and 50% of the cultures were infected with 40 PFU/cell. The virus was adsorbed 2 hr at 37 C, and excess virus was removed by washing. The fraction of virus-producing cells was then determined at daily intervals. The other 50% of the cultures were stimulated with PHA, and, at daily intervals, these cultures were also inoculated with virus at the same multiplicity of 40 PFU/cell. The virus was adsorbed at 37 C for 2 hr. Excess virus was then removed by washing, and the cells were plated for infectious centers as described in Materials and Methods.

Results of these experiments (Fig. 2) confirmed the data presented in Fig. 1. Stimulated cells

FIG. 1. Total virus yield and fraction of cells producing virus in PHA-stimulated and nonstimulated leukocytes. Symbols: ●, multiplicity of 5 PFU/cell; ○, multiplicity of 0.005 PFU/cell.

clearly exhibit a period of increased susceptibility to infection by poliovirus. The decrease in the number of cells producing virus after days 3 and 4 was not due to exhaustion of susceptible cells, since addition of virus after day 4 did not result in the same fraction of cells producing virus that was attained on day 4. To further substantiate this observation, leukocyte cultures were infected with virus, and, on day 4, the cells were washed, and 50% of the cultures were inoculated with poliovirus antibodies to prevent infection of additional cells. Infectious centers were determined daily thereafter in the cultures with and without antiserum. The fraction of infected cells was essentially the same in the presence and absence of antibody (Table 1).

FIG. 2. Fraction of cells producing virus in PHA-stimulated leukocyte cultures when virus was added at day 0 (●) or on different days after incubation (○).

TABLE 1. Effect of poliovirus antibody on the no. of cells producing virus in PHA-stimulated leukocyte cultures^a

Expt	Fraction of cells producing virus on day					
	5		6		7	
	Anti-body	No antibody	Antibody	No antibody	Antibody	No antibody
1			1/60	1/70	1/200	1/200
2	1/20	1/20	1/40	1/45	1/160	1/150

^a PHA-stimulated leukocytes were infected with poliovirus on day 0. On day 4, the cells were washed, and 50% of the cultures received poliovirus antibody to prevent cell-to-cell spread of the virus; infectious centers were determined on the subsequent 3 days. Values are the ratios of numbers of cells producing virus to the total numbers of cells in the cultures.

Replication of poliovirus in the presence of antibodies. In repeated experiments, we sometimes demonstrated that up to 20% of the cells produced virus. However, most of the leukocytes either failed to become infected or were abortively infected. The possibility was considered that the majority of leukocytes may become infected initially; however, only when these cells underwent blastogenic changes could the viral genome be expressed fully with the production of infectious virus. To test this possibility, the development of infectious centers was determined in cultures where the cell-to-cell spread of the virus was prevented with antibody.

Leukocyte cultures were prepared, and the cells were infected with poliovirus at a multiplicity of 5 PFU/cell. Virus was allowed to adsorb for 2 hr at 37 C, and excess virus was removed by washing. Cultures were resuspended in medium containing PHA. To 50% of the cultures, homologous serum containing poliovirus antibodies was added. The fraction of cells producing virus was determined daily for 5 days. No evidence of defective infection of the cells with subsequent expression of viral genome was obtained. The number of infected cells decreased in the cultures containing antibody (Fig. 3); after the third day, no cells producing virus could be detected. The data suggest that few cells in the initial cultures are susceptible to the virus and that the cells become susceptible and infected during blastogenesis.

Infectious-center assay in fresh leukocyte suspensions. When 2.9×10^3 PFU was added to 10^6 fresh leukocytes and incubated for 2 hr at 37 C, 2.7×10^3 PFU, or 93% of the virus, remained in the supernatant fluid. Poor attachment of the virus, reflected also in the scarcity of cells which were producing virus, could not be overcome by increasing the virus input. Fresh PHA-stimulated leukocyte cultures were inoculated with different

FIG. 3. Replication of poliovirus in leukocyte cultures infected on day 0 and incubated in the presence or absence of poliovirus antiserum.

multiplicities ranging from 0.1 to 200 PFU/cell. After adsorption, excess virus was removed, and the cells were plated for infectious-center assay (Table 2). There was a linear relationship between virus input and the number of cells producing virus, up to 50 PFU/cell. Additional infectious centers developed at an inoculum of 100 PFU/cell; however, the addition of 200 PFU/cell did not significantly increase the number of cells producing virus, indicating that maximal susceptibility of the fresh cells is about 1%. These studies confirm the findings of others (10) that poliovirus attaches poorly to fresh leukocytes.

Adsorption and penetration of poliovirus in leukocytes. Poliovirus at a multiplicity of 50 PFU/cell was added to 10^6 cells contained in 0.4 ml of medium. The cells used included freshly prepared macrophages, leukocytes to which PHA had been added, and leukocytes which had been stimulated with PHA and incubated for 4 days and for 7 days. The virus was allowed to adsorb for 60 min at 37 C in one sample of the cells and for 2 hr at 4 C in another sample of cells. After adsorption, excess virus was removed by washing three times with 12 ml of Hanks' BSS. Cells were resuspended in 2 ml of medium. Samples of the cells were removed, frozen and thawed twice, and assayed for virus which had adsorbed to the cells. Cells adsorbed at 37 C were then incubated for 1

additional hr at 37 C, and the penetrated virus was determined as described in Materials and Methods for infectious-center assay, except that the cells were frozen and thawed twice and assayed for infectious virus. A third sample of the cells was assayed for infectious centers.

Adsorption of poliovirus to leukocytes was greater in cultures of leukocytes which had been in culture for 4 days and also in fresh peritoneal macrophages (Table 3). Only poliovirus sufficient to infect 3 to 4% of the leukocytes was adsorbed to fresh leukocytes or to 7-day-old leukocytes. Adsorption of the virus was greater at 37 than at 4 C and the relative efficiency of adsorption to the different cells was the same at both temperatures. In leukocytes which had been stimulated with PHA and incubated for 4 or 7 days, each virion that penetrated registered as an infectious center; however, in fresh leukocytes only about 30% of the penetrated virus produced infectious centers. Peritoneal macrophages responded somewhat similarly to fresh leukocytes in that about 50% of the penetrated virus registered as infectious centers. Thus, alterations occurred in PHA-stimulated leukocytes which resulted in an increased uptake of the virus at 4 days and in virus replication in all cells in which the virus had penetrated. Cells maintained in culture for 7 days adsorbed virus to the same degree as fresh leuko-

TABLE 2. Cells producing virus in fresh leukocyte cultures after infecting with different input multiplicities^a

Expt	Cells producing virus ^a at input multiplicity (PFU/cell):									
	0.1	0.5	1	2	5	10	25	50	100	200
1		80	150		750	1,800	3,200	6,000		9,000
2	20	90	180	300	800	2,000	3,300		8,000	

^a Values are expressed as the number of cells producing virus per 10^5 leukocytes.

TABLE 3. Adsorption and penetration of poliovirus in PHA-stimulated leukocytes^a

Results	Fresh leukocytes		4-day leukocytes		7-day leukocytes		Peritoneal macrophages	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Adsorption at 37 C ^b	3.7×10^4	4.1×10^4	1.2×10^5	2.1×10^5	4.5×10^4	4.5×10^4	2.3×10^5	1.8×10^5
Adsorption at 4 C ^c	1.7×10^4	2.0×10^4	4.8×10^4	6.2×10^4	2.3×10^4	2.6×10^4	9.1×10^4	6.4×10^4
Penetrated virus ^d	1.2×10^4	1.2×10^4	3.1×10^4	3.8×10^4	6.8×10^3	7.0×10^3	6.2×10^4	3.8×10^4
Cells producing virus ^e	4.4×10^3	4.8×10^3	3.0×10^4	3.4×10^4	6.0×10^3	6.6×10^3	3.5×10^4	2.0×10^4

^a Cultures contained 1×10^6 cells/ml. Virus yield is expressed as PFU of virus per ml.

^b PFU of poliovirus adsorbed after 1 hr at 37 C.

^c PFU of poliovirus adsorbed after 2 hr at 4 C.

^d PFU of poliovirus adsorbed for 2 hr at 37 C; then extracellular virus was neutralized with poliovirus antiserum. Excess antiserum was removed, and the nonneutralized intracellular virus was assayed.

^e Ratios of number of cells producing virus to total number of cells were 1:200 in the case of fresh leukocytes, 1:30 for 4-day leukocytes, 1:150 for 7-day leukocytes, and 1:35 for peritoneal macrophages.

cytes; they maintained their ability to replicate penetrated virus. In the usual cell culture systems of mammalian cells, poliovirus replicates in 8 hr, and, at a multiplicity of 5, almost all cells produce virus (18). At a comparable virus input in leukocyte cultures, 3 to 4 days were required to obtain a maximum of virus-producing cells.

Poliovirus eclipse in leukocyte cultures. To investigate the fate of the adsorbed and penetrated poliovirus in leukocyte cultures, eclipse studies were conducted. Poliovirus at a multiplicity of 50 PFU/cell was inoculated into fresh, 4- and 7-day PHA-stimulated leukocyte cultures and into fresh cultures of peritoneal macrophages. The virus was adsorbed at 4 C for 2 hr and excess virus was then removed with three washes with cold Hanks' BSS. After the last wash, the cells were resuspended in medium, and four cultures from each group of cells tested were frozen immediately. The remaining cultures were incubated at 37 C, and duplicate cultures were frozen at various time intervals. After the entire series of samples had been collected, all cultures were frozen and thawed for an additional time and assayed for virus. For comparison, primary African green monkey kidney cells in monolayer were infected with 10 PFU/cell of poliovirus and treated similarly (Fig. 4).

Over 70% of the poliovirus eclipsed in primary African green monkey kidney cells in 45 min, after which further eclipsing did not occur. Essentially, no virus eclipsing was observed in 4 hr in the fresh leukocyte cultures. In the macrophages and the 4- and 7-day PHA-stimulated leukocytes, about 50% of the virus which adsorbed to the cells had eclipsed in 2 hr.

The duration in culture required before virus eclipsed in fresh leukocytes was determined by

FIG. 4. Eclipsing of poliovirus in fresh leukocytes, 4- and 7-day-old PHA-stimulated leukocytes, peritoneal macrophages, and primary African green monkey kidney cells.

repeating the above experiments on cultures with and without added PHA. Cultures were examined at intervals for 36 hr (Fig. 5). There was a 15% loss of the virus infectivity in the first 2 hr. Virus eclipse was not observed until the 10th hour in PHA-stimulated cultures and the 18th hour in the nonstimulated cultures. New virus appeared between 25 and 30 hr of cultivation in both stimulated and nonstimulated cultures; however, the virus yield at 30 hr was much greater in the PHA-stimulated cultures. Thus, poliovirus appears to be unable to eclipse in fresh leukocytes until the cells have remained in culture for a number of hours.

DISCUSSION

In our study of poliovirus replication in leukocytes, the original observations of Gresser and Chany could be confirmed (10), namely, that nonstimulated leukocyte cultures inoculated with an input multiplicity of less than 0.01 PFU/cell showed a lag period of 5 days before virus replication could be detected. Our data indicate that the replication of poliovirus, like that of other viruses (7), can be enhanced by stimulation of the leukocytes with PHA. The enhanced replication was due to an increase in the number of cells producing virus, the virus yield per cell being approximately 10 PFU/cell for both stimulated and nonstimulated cultures.

The relationship between viruses and various types of leukocytes has been the subject of a number of reports (reviewed by Gresser and Lang, 11). The leukocyte preparations used in our studies contained a heterogeneous cell population. Although polymorphonuclear leukocytes may show viral antigens when obtained from an infected host (27), they apparently do not support the replication of viruses *in vitro* (8). Thus, the observations reported in our study are thought to

FIG. 5. Eclipsing of poliovirus in fresh leukocyte cultures in the presence and in the absence of PHA.

represent primarily the interaction of poliovirus with monocytes and lymphocytes.

Monocytes, when cultured *in vitro*, rapidly take on the characteristics of macrophages (3). Peritoneal macrophages were found to adsorb and eclipse poliovirus. Production of new virus followed with a yield of approximately 10 PFU/cell, and cells underwent cytopathic changes.

Infection of cultures of fresh leukocytes with 200 PFU of poliovirus per cell resulted in a productive infection of less than 1% of the cells, and this small fraction of cells producing virus probably represented monocytes. Thus, the mature lymphocytes were found to be insensitive to poliovirus, a confirmation of the observations of others (8, 10). Mature lymphocytes, which seem to possess immunological memory (9), appear to be resistant to most viruses (11). In the case of poliovirus, the lack of susceptibility seems to be due to a lack of specific receptors necessary for virus adsorption (16). Virus which became cell-associated and penetrated into the cell also failed to eclipse with the usual kinetics. Data in Table 2 suggest that over 50% of the cells into which the virus penetrated were abortively infected and thus failed to yield infectious centers.

PHA stimulates blastogenic changes of lymphocytes in peripheral leukocyte preparations (25). Biochemical changes induced include changes in phosphorylation of nuclear proteins (20), acetylation of histones (23), and increased synthesis of several types of ribonucleic acid (RNA) (4, 5). Increased protein and deoxyribonucleic acid synthesis and increased activity of enzymes (14, 22) as well as pinocytotic activity (19) have been reported. These biochemical changes are associated with morphological alterations and mitosis (25). Similar changes also occur with other stimuli, including the addition of specific antigens.

Our data indicate that the stimulated lymphocytes adsorb poliovirus more efficiently, suggesting a cellular acquisition of specific receptors or increased pinocytotic activity. Increased adsorption of the virus was observed until the 4th day after addition of PHA; adsorption of the virus returned to the levels noted for fresh lymphocytes by the 7th day. Other studies in progress have shown that RNA synthesis in PHA-stimulated lymphocytes is highest 4 days after starting the cultures and coincides with the time of the highest number of infected leukocytes present in the culture.

Eclipsing of virus entails the formation of phagolysosomes (26). The lysosomal apparatus of the mature lymphocyte is poorly developed (2, 15), and it is not surprising that poliovirus eclipsed poorly in fresh cells. Although direct evidence is not available, it seems reasonable to assume that

stimulation of lysosomal enzymes by PHA accounts for the more rapid eclipsing of poliovirus in PHA-treated cells.

Less than 50% of the poliovirus that penetrated into fresh leukocytes gave rise to infectious centers, indicative of an abortive infection in a portion of the mature lymphocytes. In PHA-stimulated cultures, such abortive infections were not observed; the number of infectious centers was similar to the number of cells penetrated by the virus. Host-cell ribosomes are required for the production of new viral protein. The mature lymphocyte, which has a sparse supply of ribosomes, would not be expected to be an ideal host cell for virus synthesis (28). Since a major portion of the new RNA synthesized by lymphocytes following PHA stimulation is ribosomal RNA (4, 5), the increase in the numbers of virus-producing cells associated with lymphocyte blastogenesis may in part be explained by the formation of new ribosomal RNA which allows complete virus replication.

Recently, evidence has accumulated to indicate that a cellular immune response participates in the defense response of the host against certain viral infections. Cellular immune reactions appear to be mediated through thymic-dependent lymphocytes (1, 12, 13). Viral persistence, as observed in congenital rubella, has been postulated to result from an inhibition of lymphocyte function by the virus (6, 24). Viral persistence of poliovirus mediated through such a mechanism would be unlikely to occur *in vivo* since circulating lymphocytes are relatively resistant to infection by the virus. Conditions of high virus concentration, cell dedifferentiation, or both, which would be necessary for the virus to significantly alter lymphocyte functions are not likely to occur *in vivo*. It would appear that a unique susceptibility, such as that of embryonic lymphocytes to rubella virus, may be a prerequisite for a virus to impair lymphocyte function *in vivo*.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research contract PH 43-68-678 within the Special Virus-Leukemia Program of the National Cancer Institute, and by research grants HE 05435 from the National Heart Institute and AI 05382 from the National Institute of Allergy and Infectious Diseases.

We appreciate the technical assistance of Patricia Hill and Bobbie Curtis.

William E. Rawls was the recipient of Public Health Service Research Career Development Award 5-K3-AI25,943 from the National Institute of Allergy and Infectious Diseases.

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