Specific Role of Each Human Leukocyte Type in Viral Infections

II. Phytohemagglutinin-treated Lymphocytes as Host Cells for Vesicular Stomatitis Virus Replication In Vitro

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The mitogenic agent, phytohemagglutinin (PHA), added to human mixed leukocyte cultures and to lymphocyte cultures converted small lymphocytes into lymphoblasts and increased lymphocyte susceptibility to vesicular stomatitis virus (VSV). Maximum virus yields were 30- to 1,000-fold higher in PHA-treated than in control cultures. VSV replicated to peak titers before lymphocytes were morphologically transformed by PHA, and virus titers fell as lymphoblast destruction began. PHA neither induced significant VSV replication in polymorphonuclear leukocyte cultures, nor increased the large virus yields in monocyte cultures. The treatment of PHA with heat, digestive enzymes, rabbit anti-PHA serum and serial dilutions failed to dissociate that portion of the PHA extract responsible for the conversion of lymphocytes into virus-susceptible cells from those components responsible for leucoagglutination or lymphocyte transformation.

Numerous nonviral agents have been shown to enhance animal virus replication in monolayer cell cultures (2, 3, 8, 10, 18, 25). In human leukocyte cultures, phytohemagglutinin (PHA), an extract of the red kidney bean *Phaseolus vulgaris*, induces the replication of some viruses, such as herpes simplex (13) and mumps (4), which otherwise would not replicate, and also increases the yield of vesicular stomatitis virus (VSV) (5) and 17D yellow fever virus (Wheelock and Edelman, unpublished data), which otherwise would replicate to low titer.

PHA agglutinates human leukocytes (11, 14) and produces a variety of alterations in lymphocytes, including: (i) stimulation of ribonucleic acid (RNA) and protein synthesis (7, 11); (ii) transformation into blast cells (14, 27); (iii) stimulation of deoxyribonucleic acid (DNA) replication and mitosis (7, 14, 27) and (iv) induction of interferon production (24). PHA has been shown to enhance lymphocyte-mediated immune responses in vitro (11), and either to enhance (22) or suppress (23) immune responses in vivo.

The present study inquires into the mechanism by which PHA enhances VSV replication in human leukocyte cultures. The relationship of PHA-induced leucoagglutination, blast cell for-

mation, and enhancement of VSV replication is described, and the white cell types which undergo cytopathic alterations in PHA-treated virus-infected cultures are identified. The small lymphocyte, a cell in which VSV replicates to low titer, is shown to be transformed by PHA into a lymphoblast, a cell which can support VSV replication to high titer.

MATERIALS AND METHODS

Virus. VSV, Indiana serotype, was obtained from Frederick C. Robbins. The virus seed was prepared by replication in a PPLO (pleuropneumonia-like organism)-free L-cell strain of mouse skin fibroblasts; supernatant fluids were quick-frozen and stored at -70°C . The VSV seed contained 3.2×10^8 tissue culture infective doses, 50% (TCID₅₀) per ml, when titered in L-cell monolayer cultures.

Culture medium. Human leukocytes and the L-cell monolayers used for virus assay were cultured in Eagle's minimum essential medium (MEM) supplemented with: Tryptose Phosphate Broth, 4% (Difco); unheated fetal calf serum, 10%; sodium bicarbonate, 1.75 g/liter; glutamine, 1%; penicillin and streptomycin. All cell-culture vessels were gassed with 5% CO₂ in air and incubated stationary at 37 C.

Preparation of mixed leukocyte cultures. The venous blood of healthy adults was drawn into plastic disposable syringes and then placed in glass

vessels containing 0.2 ml of phenol-free heparin (Upjohn) for each 10 to 14 ml of blood. After incubation at 37 C for 30 to 60 min, the leukocyte-rich plasma upper phase was aspirated and centrifuged at $225 \times g$ for 10 min to sediment the leukocytes. The plasma supernatant fraction, now containing principally platelets, was aspirated. The cell pellet in the bottom of the tube was gently resuspended in a large volume of phosphate-buffered saline (PBS), centrifuged again, and the remaining plasma, heparin, and platelets were drawn off with the supernatant fluid. The washed leukocytes were then resuspended in culture medium. The yield of leukocytes from 50 ml of venous blood averaged 150×10^6 cells; more than 99.5% were viable. The procedures employed in obtaining leukocyte cultures did not significantly alter the cell differential count from that of the donor's whole blood, by using the morphological criteria outlined by Wintrobe (26). Erythrocytes were present, and their quantity varied from one-tenth to 10 times the leukocyte concentration. Platelets were absent, having been removed during preparation of the leukocyte suspensions. Cell cultures which were prepared directly from peripheral blood as described above are designated mixed leukocyte cultures. In mixed leukocyte cultures treated with PHA, the identification of blast cells and of lymphocytes in transformation was based on the morphological criteria of Yoffey et al. (27), and both cell types will be referred to collectively as "blast cells" or "lymphoblasts" to distinguish them from small lymphocytes (see Fig. 2). Unless indicated otherwise, a given experiment was performed with freshly prepared cells from a single healthy adult donor, although donors of human leukocytes varied from experiment to experiment.

Determination of cell viability. Immediately after being placed in culture, and at selected intervals during the course of experiments described below, the leukocytes were counted in a hemocytometer chamber employing an erythrosin B vital dye exclusion technique (15). Each count represented the identification of 100 to 250 cells which excluded uptake of erythrosin B vital dye. 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 leukocyte resuspended 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 (15×15 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 coverslips were identified as to cell type. Three to five separate areas on each cover slip were examined to minimize the effect of cell aggregation and unequal cell distribution. Leukocytes showing evidence of cell damage were not counted, and the rare leukocyte that appeared intact but not identifiable as to cell type was not included in the differential count. 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 by using silicone-treated glass-bead columns. The white blood-cell types were separated on the basis of their differential adherence to and elution from siliconized glass bead columns (17). Lymphocytes did not adhere to glass beads, whereas polymorphonuclear (PMN) leukocytes and monocytes did adhere and were selectively eluted by buffered ethylenediaminetetraacetic acid (EDTA) in saline. After removal from a glass bead column, the cell fractions were washed once to remove autologous plasma or EDTA, resuspended in culture medium, adjusted to a cell concentration of 10^6 cells/ml, and samples of each of the suspension cultures were taken for differential leukocyte counts. Loss of cell viability during column separation was less than 0.5%.

Virus growth curves and infectivity assay. After VSV inoculation into leukocyte cultures at a virus-to-cell multiplicity of 1, the cultures were placed in a stationary position for 1 hr at 37 C to allow virus absorption. The leukocytes were then washed by 1 cycle of centrifugation at $225 \times g$ for 10 min; the cellular pellets were resuspended in fresh culture medium at the original volume and incubated at 37 C. PHA was not added to the cultures that had earlier received PHA, since preliminary studies indicated there was no further virus enhancement when PHA was added again after the cell wash. At timed intervals, the sedimented cells and cells adhering to the glass vessels were resuspended by pipetting, and 1-ml samples of the infected cultures were removed, frozen rapidly, and stored at -70 C. Immediately before virus assay, the frozen samples were thawed and frozen rapidly twice to disrupt cells, a procedure which produced no detectable loss of VSV infectivity. Infective virus was assayed by the tube dilution method in monolayers of human diploid fetal lung cells or in the L-cell strain of mouse skin fibroblasts in screw-cap glass culture tubes (16×125 mm). Both cell lines are equally susceptible to VSV; PHA does not influence the replication of VSV in the cell lines used for virus assays (5). Titrations employed five to eight tubes per 10-fold dilution, and the 50% end point was calculated by the method of Reed and Muench (19). Differences in titer of more than 0.5 $\log_{10}$ (3.2-fold) were considered to be significant.

Assay of human interferon. Samples of leukocyte cultures obtained for interferon assay were centrifuged to remove cells, and the culture medium was stored at 4 C. Interferon was considered to be present when a 1:2 dilution (or more) of the culture medium protected monolayer cultures of human fetal lung fibroblasts against Sindbis virus challenge (24).

PHA. Lyophilized commercial PHA-P (Difco lot #475973) was dissolved in PBS (10 mg/ml), sterilized by filtration, and stored at 4 C. A freshly prepared stock of PHA was used every 4 weeks. A 25- to 50- μ g amount of PHA in an 0.05-ml volume was added to each 1-ml amount of leukocyte suspension containing 10^6 cells/ml. The time of addition of PHA with respect to VSV inoculation was not

crucial; virus enhancement occurred if PHA was added either with VSV or 5 days before virus inoculation.

Preparation and titration of anti-PHA serum. An adult white female rabbit was injected both subcutaneously and intramuscularly with 25 mg of PHA in saline and complete Freund's adjuvant, once each week for 5 weeks; one-half of the final inoculating dose, free of adjuvant, was administered intravenously. Serum—obtained 1 week after the last injection—was heated (56 C, 30 min) to remove leucoagglutinating activity. Serial dilutions of serum were then incubated in PHA (50 μ g of PHA/ml of culture medium) at 37 C for 1 hr; leukocytes were added to the incubation mixture (10⁶ cells/ml, final dilution), kept overnight at 4 C, and the white cell aggregates were then graded as to size and number. An antiserum dilution of 1:40 inhibited leucoagglutination by PHA.

Data showing the effect of anti-PHA serum on PHA activity (Table 1) were obtained in the following manner. Anti-PHA serum (0.2 ml) was incubated with 2.0 ml of a solution containing 50 μ g of PHA/ml of culture medium for 1 hr at 37 C. The flocculent precipitate that formed was then removed by centrifugation (5,000 rev/min for 30 min), the supernatant fluid added to an equal volume of mixed leukocytes in culture medium, and the residual PHA activity determined as previously described. PHA preincubated with heat-inactivated serum from a nonimmunized rabbit served as a control.

RESULTS

Correlation of the leucoagglutinating, blastogenic, and VSV-enhancing activities of PHA in mixed leukocyte cultures. To elucidate the mechanism of PHA-induced enhancement of VSV replication in human mixed leukocyte cultures (5), a series of experiments was performed to correlate the various effects of PHA on leukocytes and on virus replication.

PHA was added in 0.5-log₁₀ (3.2-fold) increments to a series of mixed leukocyte cultures containing 10⁶ cells/ml. The degree of leucoagglutination (graded 0 to + + + +, shown on the abscissa, Fig. 1) was based on the number and size of microscopic white-cell aggregates in uninfected cultures incubated at 37 C for 16 hr after the addition of PHA. The uninfected cultures were harvested 64 hr after the addition of PHA, and the percentage of blast cells (also shown on the abscissa) was calculated from a total count of 1,000 cells from each culture. Additional leukocyte cultures, prepared in an identical fashion to cultures described above, received VSV 16 hr after the addition of PHA, and the cultures were washed 1 hr after virus inoculation (*see Materials and Methods*). The virus titers expressed on the ordinate in Fig. 1 are those present at 57 hr after VSV inoculation, which is a time on the virus growth curves when the differences between VSV yields in PHA-treated and control cultures are expected to be at a maximum (5).

With decreasing doses of PHA, starting at a concentration of 50 μ g/ml of culture, there was a linear decrease in PHA-induced VSV enhancement, leucoagglutination, and blast-cell formation (Fig. 1); all of these effects disappeared at the same terminal dilution. The lower limit of PHA activity was detected at the concentration of 0.5 μ g of PHA/ml. Additional studies correlating the dose of PHA with the formation of interferon in mixed leukocyte cultures showed that interferon was detectable in leukocyte cultures treated with a minimum of 1.6 μ g of PHA/ml in one experiment and with a minimum of 5.0 μ g of PHA/ml in a second experiment.

In addition to altering the concentration of the

FIG. 1. Correlation of the leucoagglutinating, blastogenic, and vesicular stomatitis virus (VSV)-enhancing activities of phytohemagglutinin in human leukocyte cultures.

crude PHA extract, other means were utilized to eliminate selectively one or more of the PHA-induced effects. Table 1 summarizes the effect of treatment of PHA with heat, anti-PHA serum, and several digestive enzymes on PHA-induced leukoagglutination, blastogenesis, and virus enhancement in mixed leukocyte cultures. The three PHA-induced effects were destroyed by heat (88 to 90 C for 20 min) and were neutralized with serum from a PHA-immune rabbit (37 C for 1 hr), whereas heat (56 C for 1 hr), trypsin, and nucleases had no effect. The ability of PHA to induce interferon was abolished by prior treatment of PHA with heat (88 to 90 C for 20 min) and with anti-PHA serum. These results suggest that blast-cell transformation, leukoagglutination, and enhancement of VSV replication are interrelated events.

Effect of VSV on cell morphology and survival in PHA-treated mixed leukocyte cultures. To elucidate further the possible relationship between virus enhancement and blast-cell formation, the effect of VSV on leukocyte morphology and survival was studied, and the cell type or types destroyed as a consequence of infection were determined (Table 2).

A freshly prepared mixed leukocyte culture containing 2×10^6 cells/ml was divided into four cultures. One was inoculated with PHA and VSV simultaneously, while a companion culture received only PHA; the two remaining cultures were treated with culture medium instead of PHA (controls), and one of these cultures was

inoculated with VSV. After 1 hr of incubation at 37 C, the cells were washed once and the cultures were incubated in a stationary position at 37 C for 3 additional days to allow adequate time for formation of lymphoblasts and to permit VSV to reach maximum titer (5). Three days after VSV inoculation, samples of each culture were taken for cell viability counts (cell survival), for differential counts (cell morphology), and for virus assay. Differential counts were performed on a total of 500 cells from each culture. Although leukoagglutination did not permit accurate cell counts 1 day after the addition of PHA, by 2 or 3 days most of the cell aggregates had partially dissociated and reproducible cell counts could be obtained.

Lymphoblast transformation was noted after an incubation period of 72 hr in all cultures (Table 3). In the uninfected PHA culture, there were 20 times more lymphoblasts than in the infected PHA culture, and 40 times more lymphoblasts than in either of the control cultures. A comparison of the two PHA-treated cultures revealed a 95% loss of blast cells in the virus-infected culture at 72 hr; this loss ranged between 75 and 97% in additional experiments. There was no detectable loss of lymphoblasts in the VSV-infected control culture as compared with the uninfected control culture. In contrast to the marked destruction of lymphoblasts in the infected PHA culture, there

TABLE 1. *Influence of heat, antiphytohemagglutinin (PHA) serum, and digestive enzymes on the activity of PHA*

Treatment of PHA	PHA activity		
	Leuko-agglutination	Blast-cell formation	VSV ^a enhancement
Untreated	+ ^b	+	+
Heat at 56 C, 1 hr	+	+	+
Heat at 88-90 C, 20 min	0	0	0
Anti-PHA serum (37 C, 1 hr)	0	0	0
Trypsin, 0.1 mg/1 mg of PHA (37 C, 1 hr)	+	+	+
Deoxyribonuclease, 5 µg/1 mg of PHA (37 C, 1 hr)	+	+	+
Ribonuclease, 5 µg/1 mg PHA (37 C, 1 hr)	+	+	+

^a VSV = vesicular stomatitis virus.

^b Plus (+) = no reduction in activity; zero (0) = absence of detectable activity after treatment.

TABLE 2. *Effect of vesicular stomatitis virus (VSV) on cell survival in mixed leukocyte cultures*

Treatment	Total no. of each cell type ^a /ml of culture $\times 10^4$				Virus titer, (TCID ₅₀ /ml) ^b
	0 hr		72 hr		
	P + L + M	B	P + L + M	B	
Control					
Uninfected	2,000	0	760	5.3	
Infected	2,000	0	710	6.4	2.3×10^4
PHA ^c					
Uninfected	2,000	0	290	210	
Infected	2,000	0	263	11	6.8×10^5

^a Total number of each cell type was obtained by multiplying the percent differential count for each culture by the total number of viable leukocytes in that culture at either the time of VSV inoculation (0 hr) or at 72 hr after VSV inoculation (72 hr). Symbols P + L + M = combined white cell fractions of polymorphonuclear leukocytes (P), lymphocytes (L), and monocyte-macrophages (M). Lymphoblasts (B) are listed separately.

^b Assayed 72 hr after virus inoculation.

^c Culture was inoculated with phytohemagglutinin (PHA) and VSV simultaneously.

was only a minimal loss of leukocytes in the combined nonblast cell fraction (P + L + M; Table 2) of both virus-infected cultures. This cell loss, equivalent to a 6.5% and a 9.3% reduction, respectively, in the control and PHA-treated cultures, could be attributed to cytopathic effects in the monocyte-macrophage fraction of the mixed leukocytes (6). PHA treatment was associated with a 30-fold enhancement of the virus titer compared to the control.

Figure 2 shows lymphocyte transformation in mixed leukocyte cultures treated with PHA for 3 days; Fig. 3 illustrates cytopathic alterations in a similar culture 3 days after the simultaneous inoculation of both VSV and PHA. The small lymphocytes and polymorphonuclear leukocytes were well preserved in the infected culture (Fig. 3), but there were fewer intact transformed lymphocytes present as compared with the uninfected culture (Fig. 2). Monocyte-macrophages are not shown in either Fig. 2 or Fig. 3.

The results presented above suggest that, as a consequence of infection, PHA-stimulated lymphocytes were destroyed either before their transformation into blast cells or shortly thereafter, and that increased virus yields in these PHA-treated mixed cultures may be attributed to VSV replication in the transforming lymphocyte fraction. Studies were next undertaken to elucidate the temporal relationship between

VSV replication and lymphoblast production and destruction.

Duplicate mixed leukocyte cultures were prepared, treated with PHA for several minutes, and then one culture was inoculated with VSV. At 24, 48, 72, and 96 hr after VSV inoculation, determinations were made of the total numbers of lymphoblasts present in each of the cultures by utilizing methods outlined above. A virus growth curve was constructed from samples of the VSV-infected cultures. In the uninfected culture, lymphocyte transformation was first detected at 48 hr after the addition of PHA, and increasing numbers of blast cells appeared between 48 and 96 hr (Fig. 4). By contrast, in the VSV-infected culture, fewer blast cells were detected at 48 hr, and there was a steady decline in the number of transformed cells over the subsequent 48 hr. Excess blast-cell loss in the VSV-infected culture (as compared with the control culture) was first apparent 24 hr after peak virus titers were reached, and virus titers declined as the loss of blast cells increased between 48 and 96 hr.

These results indicate that VSV can replicate peak titers before lymphocytes are morphologically transformed into lymphoblasts, and that virus titers fall shortly after lymphoblast destruction begins.

Effect of PHA on the replication of VSV in lymphocyte cultures. The results of the studies presented thus far clearly implicated the PHA-stimulated lymphocyte in the enhancement

FIG. 2. Photomicrograph of a noninfected leukocyte culture treated with phytohemagglutinin for 3 days; lymphocytes (L), lymphocytes in transformation (T), and lymphoblasts (B). No attempt is made in the text to distinguish between lymphoblasts and transforming lymphocytes, and both cell types are referred to collectively as "blast cells" or "lymphoblasts." Wright's stain, $\times 1,924$.

FIG. 3. Photomicrograph of a mixed leukocyte culture 3 days after inoculation with vesicular stomatitis virus and phytohemagglutinin; lymphocytes (L), polymorphonuclear leukocytes (P), and disrupted leukocytes (X) which cannot be precisely identified. No macrophages or blast cells can be seen. Wright's stain, $\times 1,924$.

FIG. 4. Temporal relationship between virus replication, lymphocyte transformation, and lymphoblast destruction in mixed leukocyte cultures treated with phytohemagglutinin (PHA). VSV = vesicular stomatitis virus.

phenomenon previously noted in mixed leukocyte cultures (5). The correlation of virus enhancement and blast-cell transformation, together with the observation that the large majority of PHA-stimulated lymphocytes undergo cytopathic alterations in infected cultures, suggested that VSV replicates in this cell type. Therefore, interest was focused next on the effects of PHA in cultures of small lymphocytes.

Lymphocyte cultures were prepared by passage of mixed leukocytes through silicone-treated glass-bead columns according to the technique outlined in Materials and Methods. Duplicate lymphocyte cultures were composed of 99.7% small lymphocytes and 0.3% PMN leukocytes. In addition, duplicate mixed leukocyte cultures containing 30% lymphocytes, derived from a sample of the same batch of buffy-coat cells that served as the source of lymphocyte cultures, were established without passage through the glass-bead column. After the cultures were adjusted to 10×10^6 cells in a 10-ml volume, PHA was added to one culture of each of the two paired sets of cultures. After 16 hr of incubation at 37 C, a sample was obtained from each culture for interferon assay. VSV was then inoculated into the four leukocyte cultures, and virus growth curves were constructed as described above.

Figure 5 illustrates the replication of VSV in paired cultures of lymphocytes and of mixed leukocytes, in the presence and absence of PHA. In the untreated mixed leukocyte culture, a peak virus titer of $10^{5.15}$ TCID₅₀/ml was reached at 15 hr after virus inoculation, whereas, in the

PHA-treated mixed leukocyte culture, a maximum titer of $10^{7.15}$ TCID₅₀/ml was achieved at 24 hr, which represents a 100-fold increase in virus yield. Significantly, the maximum virus titer in the PHA-lymphocyte culture ($10^{7.50}$ TCID₅₀/ml) was equal to, or slightly greater than, the maximum titer in the PHA-mixed leukocyte culture, and the time course of virus replication in both PHA-treated cultures were similar. There was no significant VSV replication in the non-PHA-treated lymphocyte culture. New virus was first detected 6 hr after VSV inoculation in the untreated mixed culture and after 8 hr in both PHA cultures. The extension by 2 hr of the viral eclipse period in PHA-treated cultures, together with additional studies showing extensions of as long as 10 hr in PHA cultures, may be attributed to the viral inhibitory effect of interferon produced by lymphocytes in response to PHA. Interferon was detected in both PHA-treated cultures but not in the untreated cultures. It is evident, however, that the level of antiviral activity was insufficient to prevent VSV from replication to high titers in PHA-treated lymphocytes.

VSV enhancement in PHA-treated mixed leukocyte cultures can be attributed to the propagation of VSV in PHA-stimulated lymphocytes, and this cell fraction is incapable of supporting significant virus replication without PHA (Fig. 5).

FIG. 5. Effect of the addition of phytohemagglutinin (PHA) on vesicular stomatitis virus (VSV) replication in both human mixed leukocyte cultures (containing 30% lymphocytes) and lymphocyte cultures (containing 99.7% lymphocytes). Each culture contained approximately 10^6 leukocytes at the time of virus inoculation.

Previous studies have shown that, in the absence of PHA, VSV replicates principally in the monocyte-macrophage fraction of mixed leukocyte cultures (6).

Effect of PHA on VSV replication in monocytes and polymorphonuclear leukocytes. To exclude the participation of the other circulating white blood cell types in the enhancement phenomenon, predominantly pure cultures of PMN leukocytes and of monocytes were prepared by using siliconized glass-bead columns (17). The experimental procedures were identical to those described above. Monocyte cultures contained 97% monocytes and 3% PMN leukocytes, and the PMN leukocyte cultures were composed of 95% PMN leukocytes and 5% small lymphocytes.

Figure 6 illustrates the effect of PHA on VSV replication in nearly pure cultures of both monocytes and PMN leukocytes. VSV replicated to high maximum titer in the untreated monocyte culture ($10^{7.15}$ TCID₅₀/ml). The slightly higher maximum titer in the PHA-monocyte cultures ($10^{7.50}$ TCID₅₀/ml) was within the error of titration. There was no significant virus replication in the PMN leukocyte cultures, whether or not the cultures were treated with PHA. PHA neither induced virus replication in PMN leukocyte cultures nor enhanced virus yields in monocyte cultures. Interferon was not detected in either the PHA-treated monocytes or PMN leukocyte cultures.

DISCUSSION

The present study demonstrates that significant VSV replication does not occur in small lympho-

FIG. 6. Effect of the addition of PHA on VSV replication in human monocyte cultures (97% monocytes) and in polymorphonuclear (PMN) leukocyte cultures (95% PMN leukocytes). Virus yield is expressed in terms of TCID₅₀/ml of culture containing approximately 10^6 leukocytes at the time of virus inoculation.

cytes in vitro except when these cells are stimulated by PHA. Furthermore, the previously reported enhancement of VSV yields in human mixed leukocyte cultures by PHA (5) can be ascribed to the induction of virus replication in lymphocytes that comprise approximately 30% of the total cell population of mixed leukocyte cultures.

The addition of PHA failed to induce significant VSV replication in PMN leukocyte cultures or to increase the high virus yields in monocyte cultures. These findings give further support to the concept that PHA-stimulated lymphocytes play the key role in the virus enhancement phenomenon. The PHA-mediated conversion of lymphocytes into cells capable of supporting VSV replication occurs within 8 to 16 hr, since newly synthesized virus is first detected 8 to 16 hr after the simultaneous inoculation of lymphocyte cultures with VSV and PHA. Peak VSV titers are reached at 24 to 48 hr, before morphological changes indicative of lymphocyte transformation have occurred.

The process by which PHA converts a non-productive virus infection in lymphocyte cultures into a highly productive one remains to be elucidated. Several factors may contribute, dependent upon PHA and operating within the first 8 to 16 hr after virus inoculation. The more obvious possibilities include: enhanced virus absorption or penetration into lymphocytes, or a change in cell metabolism that permits VSV replication to proceed in lymphocytes already infected with virus. Since an increased rate of RNA and protein synthesis occurs within 2 to 10 hr after lymphocytes are stimulated with PHA (11, 7), and DNA synthesis is increased only after 24 to 48 hr (27, 21), VSV replication may be associated with the earlier change in RNA or protein metabolism.

The percentage of cells producing infective virus particles in PHA-treated lymphocyte cultures is not known. The observation that enhanced VSV replication is associated with destruction of 75 to 97% of the lymphoblasts in PHA-treated leukocyte cultures is, on the one hand, compatible with the hypothesis that virtually all blast cells produced infectious virus particles prior to their destruction. On the other hand, the destruction of blast cells in VSV-infected cultures may have been the result of a poorly productive cytocidal infection in the large majority of infected, PHA-stimulated lymphocytes, and only a small percentage of the blast cells destroyed may have been responsible for the large yields of VSV.

PHA has recently been shown to contain at least three physically distinct and active compo-

nents: a leucoagglutinin (16, 20), a factor which induces RNA synthesis in lymphocytes (16, 20), and a factor which induces DNA replication (mitogenic factor; 16). In the present study several methods were employed in an attempt to isolate a possible fourth factor responsible for enhancement of VSV. Initially, the concentration of PHA was altered in the hope of diluting out its blastogenic (possible RNA-synthesizing factor) and leucoagglutinating activities. Next, anti-PHA serum was employed to separate the active, antigenic factors of PHA (1), from possible active, nonantigenic components. Finally, PHA was exposed to heat, trypsin, ribonuclease, and deoxyribonuclease. It was found that the portion of the PHA extract responsible for converting lymphocytes into virus-susceptible cells could not be dissociated from the components responsible for leucoagglutination or blast-cell transformation. Thus, virus enhancement may be intimately related to the activity of one of the aforementioned components of PHA (20). The complete separation of PHA into component parts has not been achieved, and the existence of as yet unrecognized factors responsible for the induction of virus replication in human lymphocytes cannot be excluded.

A problem raised in the present study and by past reports (13, 4) concerns the PHA-related specificity of the virus-enhancement phenomenon. Is enhancement of VSV replication peculiar to the interaction of PHA and lymphocytes, or will any lymphocyte mitogen enhance virus replication? Two mitogenic substances, rabbit anti-human lymphocyte serum (ALS; 9) and streptolysin (SLS; 12) have been tested for their VSV-enhancing properties in human lymphocyte cultures. Both ALS (6a) and SLS (*in preparation*) significantly enhanced virus replication; but SLS, in contrast to both ALS and PHA, enhanced virus replication in lymphocytes without associated leucoagglutination. Thus, the enhancement of VSV replication in lymphocytes is not peculiar to PHA, nor does it depend upon lymphocyte leucoagglutination; rather, it is probably more generally related to stimulation of lymphocyte metabolism.

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