Herpes Simplex Virus Replication in Human Lymphocyte Cultures Stimulated with Phytomitogens and Anti-Lymphocyte Globulin[1] (36941)

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A number of viral agents have now been shown to replicate in cultures of human lymphocytes (1–7). With certain of these agents replication is enhanced if the cultures are stimulated to blastogenesis. Such enhancement has been reported with vesicular stomatitis and polioviruses in cultures stimulated by phytohemagglutinin (PHA) (8–10), with poliovirus in cultures induced to blastogenesis by antigens to which the donor has been previously sensitized (11), and with vesicular stomatitis virus in cultures stimulated by anti-lymphocyte serum (12).

Previous studies in this laboratory have demonstrated replication of herpes simplex virus, a DNA virus, in lymphocyte cultures stimulated by PHA. In the absence of this mitogen, however, no viral replication was observed (13). These findings were subsequently confirmed by electron microscopy which revealed that viral replication in PHA-stimulated lymphocyte cultures appeared limited to the transformed cells (14).

Since blastogenesis induced by PHA appeared to be a prerequisite for replication of herpes simplex virus, experiments were initiated to determine whether viral replication would also occur in lymphocyte cultures stimulated to blastogenesis by other mitogens. In this report, replication of herpes simplex virus in PHA-stimulated lymphocyte cultures is compared with replication of this agent in cultures stimulated by three other mitogens, rabbit anti-human lymphocyte globulin (ALG) (15, 16), pokeweed mitogen (PWM) (17), and concanavalin A (Con A) (18).

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Materials and Methods. Preparation of lymphocyte cultures. Venous blood was collected aseptically from adult donors directly through tubing into a 250-ml Erlenmeyer flask, containing 2000 units of preservative-free sodium heparin (Panheprin, Abbott Laboratories) for each 100 ml of blood to be drawn. The blood was immediately transferred into a series of 15-ml screw-cap centrifuge tubes. The tubes were capped and allowed to stand at 37° for at least three hours. Suspensions containing up to 90% lymphocytes were obtained by withdrawing and gently centrifuging the upper one-half of two-thirds of the plasma layer at 150*g* for ten minutes. The cells were washed twice in tissue culture medium 199 (Grand Island Biological Co.), supplemented with 20% inactivated calf serum and containing 100 units of penicillin and 100 μg of streptomycin per ml. Differential and total cell counts were performed and the cells were resuspended in the above medium to give a final lymphocyte concentration of 2 to 2.4 $\times$ 10^6 lymphocytes per ml. Cultures were prepared by distributing this suspension in 0.5-ml amounts in 12-ml conical screw-cap tubes. The volumes of the lymphocyte cultures were adjusted to contain between 1 and 1.2 $\times$ 10^6 lymphocytes per ml by adding 0.5 ml of media containing the mitogen to be tested. Cultures similarly prepared but lacking mitogens were included as controls. Duplicate cultures were employed for all experiments. All cultures were incubated for three days at 37° in an atmosphere of 5% CO_2 prior to the addition of virus.

Mitogens. These preparations were obtained from the following sources: PHA—

prepared in this laboratory (19); PWM—Grand Island Biological Co.; ALG—kindly supplied by Dr. S. Cooperband, Boston University School of Medicine; Con A–Miles Laboratory. The mitogens were used in amounts previously determined to produce maximum DNA synthesis in this system, *i.e.*, 63 μg/0.5 ml for PHA; 18 μg/0.5 ml for PWM; 0.02 ml/0.5 ml for ALG; 115 μg/0.5 ml for Con A.

Virus. Herpes simplex virus (HSV), Rodanus strain, was originally recovered from the lung of a patient with eczema herpeticum and had undergone multiple passages in our laboratory, including 19 passages in human amnion cells. The infectivity titer of the virus suspension used in these experiments was $10^{6.6}$ to $10^{7.2}$ TCD_{50}/0.2 ml for human amnion cells.

Inoculation of virus. Following three days incubation, the media from the lymphocyte cultures were removed and discarded and the cells were resuspended to original volumes (1 ml) in fresh media containing HSV ($10^{4.6}$ to $10^{5.2}$ TCD_{50}/0.2 ml). After two hours adsorption at 37°, with periodic shaking to insure maximum cell–virus contact, the fluid was removed from all cultures and the cells were washed twice and resuspended to original volume in fresh media. Mitogens were not replaced. Duplicate samples of the resuspended cultures were either pooled and frozen or frozen separately at this point and stored at —65° in a Revco freezer for subsequent virus assay to provide baseline titers for virus replication. The remainder of the cultures were incubated at 37° in an atmosphere of 5% CO_2. At intervals of 7, 24, 48, 72, and 96 hr after virus addition they were similarly frozen and stored for later virus assay.

Determination of lymphocyte transformation. The extent of DNA synthesis was determined by the degree to which (^{3}H methyl) thymidine was incorporated into cellular DNA (20). Lymphocyte cultures, after virus addition and washing, were labelled for 24 hours with 0.1 μc ^{3}H thymidine per ml of media at 3, 4 or 5 days after mitogenic stimulation (*i.e.*, 0, 1, or 2 days after addition of virus). The cultures were then washed with medium 199 containing 20% calf serum and precipitated with 10% trichloroacetic acid (TCA) at 4° for 30 min. The resulting precipitates were washed twice with 10% TCA and then hydrolyzed at 90° to 100° for 30 min in 0.1 *M* oxalic acid. The hydrolysates were added to 10 ml of liquid scintillation fluid (5 gm PPO, 100 gm naphthalene/liter of dioxane) and counted in a liquid scintillation counter (Beckman). The extent of blastogenesis was measured by determining the percent of viable large cells in the hemocytometer just prior to sacrificing the cultures for virus assay.

Viral assay. Infectivity titers of HSV were determined in trypsinized cultures of primary human amnion cells maintained in bovine amniotic fluid media (21). Prior to preparation of dilutions, samples were quickly thawed and frozen three times to disrupt the cells and release intracellular virus. Serial 10-fold dilutions of both control and mitogen-

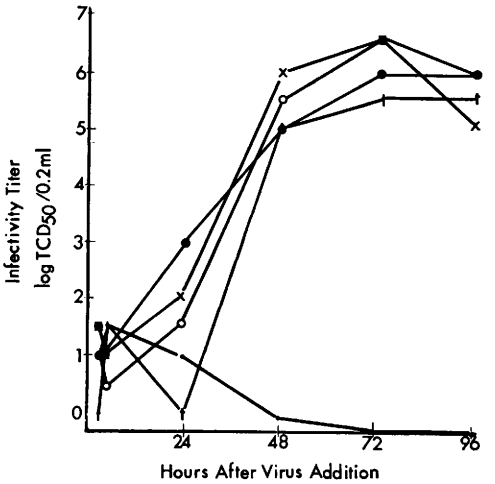

FIG. 1. The effect of mitogenic stimulation of human lymphocyte cultures on the replication of herpes simplex virus. All stimulated cultures were incubated with the appropriate mitogen for three days prior to the addition of virus. The initial inoculum of virus was $10^{4.6}$ to $10^{5.2}$ TCD_{50}/0.2 ml for human amnion tissue culture. Cultures were incubated for two hours with virus and unabsorbed virus removed by washing the cells (See Materials and Methods for details). Unstimulated control (•—•); PHA stimulated (x—x); PWM stimulated (O—O); Con A stimulated (●—●); ALG stimulated (+—+).

stimulated cultures were prepared in Hanks' BSS and 0.2 ml of each dilution inoculated into two or more human amnion cultures tubes. These tubes were kept stationary at 37° and observed for 10 days for cytopathogenic effects. Titers were calculated by the Reed and Muench method and expressed as the log TCD_{50}/0.2 ml.

Results. With the technics described, each of the mitogens tested was shown conclusively to stimulate DNA synthesis and to support the replication of herpes simplex virus in lymphocyte cultures. The viral titers with PWM, ALG and Con A were approximately equivalent to those obtained with PHA. Representative data are plotted in Fig. 1.

Maximum virus titers with all four mitogens were reached between 48 and 72 hr after virus inoculation and persisted at about peak levels for 48 hr or more. No virus replication occurred in the non-mitogen-treated cultures.

Table I demonstrates that the DNA synthesis with all four mitogens tested was generally equivalent with respect to maximum levels of DNA and the time after stimulation that this maximum was attained. The only difference among the mitogens appeared to be the rapidity with which the PWM-stimulated cultures decreased in terms of total counts. When lymphocyte transformation was measured by morphology, however, a substantial difference in the degree of blastogenesis was observed. PHA was most active and PWM least active in this respect (Table I).

Discussion. We have demonstrated the replication of herpes simplex virus in human lymphocyte cultures stimulated with PHA, PWM, Con A and ALG. It is not clear at

TABLE I. Comparison of DNA Synthesis, Blastogenic Transformation, and Herpes Simplex Virus Replication in Human Lymphocyte Cultures Stimulated with Various Mitogens.

Mitogen	Days after stimulation[a]	DNA synthesis (CPM)[b]	Virus titer[c]	% Transformation
None	3	ND	$10^{0.5}$	0
	4	280	$10^{0.5}$	0
	5	430	$10^{0.0}$	0
	6	440	0	0
PHA	3	ND	$10^{1.0}$	25–30
	4	7000	$10^{2.0}$	40–50
	5	6510	$10^{6.5}$	50–60
	6	5400	$10^{6.5}$	50–60
PWM	3	ND	$10^{1.0}$	2–5
	4	6950	$10^{1.5}$	5–25
	5	4300	$10^{5.5}$	10–20
	6	1400	$10^{6.5}$	1–10
Con A	3	ND	$10^{1.5}$	10–20
	4	6410	$10^{3.0}$	20–25
	5	7310	$10^{5.0}$	20–25
	6	5400	$10^{6.0}$	20–25
ALG	3	ND	$10^{1.0}$	20–25
	4	6500	$10^{0.0}$	40–50
	5	5800	$10^{4.5}$	40–50
	6	5800	$10^{5.5}$	25–50

[a] Cultures were labelled at 3, 4, or 5 days after stimulation with the appropriate mitogen and sacrificed after 24 hr.

[b] The CPM are based on comparable experiments with each mitogen.

[c] Cultures were stimulated for 3 days with the appropriate mitogen prior to the addition of virus; *i.e.*, day 3 is day 0 in the virus assay; day 4 is day 1, *etc.*

present how these mitogens act or whether they have similar mechanisms. Kay and Oppenheim have shown that the binding sites on the lymphocyte membrane differ for each mitogen (22). Whatever the prerequisite(s) for viral replication may be, it appears from our results that PHA is not unique in this respect.

All four mitogens produced transformation in human lymphocyte cultures. PHA and ALG were the most active when transformation was measured by cellular morphology. When DNA synthesis was used as a measure of stimulatory activity, however, the mitogens were more equivalent in activity. The peaks in DNA synthesis, as measured by ^{3}H thymidine incorporation, occurred at approximately the same time after stimulation and were comparable with all four mitogens.

The relationship between DNA synthesis and blastogenesis may not be a direct one, and the essential step in the transformation of the small lymphocyte which is required for HSV replication is not clear.

For example, Salzman *et al.* demonstrated that blastogenesis could occur in the absence of DNA synthesis, following PHA stimulation (23). Using the metabolic inhibitor, 5-fluorodeoxyuridine (FUDR), the same percentage of cells underwent morphological transformation in the presence of the inhibitor and were in almost all respects similar to those obtained in the absence of this analog. Morphological transformation was also obtained without accompanying RNA and DNA synthesis by using sub-lethal doses of sonication (24). These cells were also similar morphologically to those obtained following PHA stimulation. Polgar and Kibrick demonstrated that not all the cells which became blastoid following mitogenic stimulation synthesize DNA (25). In comparing PHA, PWM and Con A stimulation, Smith *et al.* found that while PHA induced maximal morphological transformation, it was not always associated with maximal uptake of ^{3}H thymidine; a higher uptake was observed with Con A (26).

Our results demonstrate that PWM-stimulated lymphocytes undergo little morphological transformation (5%–25% compared to 50%–60% with PHA) yet synthesize DNA in amounts comparable to PHA. This seems further evidence that not all the cells which become blastoid synthesize DNA.

Normal lymphocytes, when stimulated by PHA, show substantial increases in DNA polymerase activity over a two-to-three-day period following stimulation. This precedes blast-cell formation and mitosis by 24 hr (27). Increased DNA polymerase activity is likely an essential preliminary to increased DNA synthesis in cells responding to mitogenic stimulation and may also be essential to the replication of DNA viruses, such as HSV.

It thus remains to be determined whether the participation of the mitogens in virus replication in the lymphocyte is due to the metabolic alterations singularly, or perhaps also to structural alterations such as membrane changes.

Summary. Replication of herpes simplex virus in lymphocyte cultures stimulated by PWM, Con A, and ALG is compared with the replication of this virus in PHA-stimulated lymphocytes. The resulting virus titers are similar with all the mitogens tested and appear to be dependent on the amount of DNA synthesized by the cells rather than on blastogenesis.

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