Viral Replication in Cultures of Phytohemagglutinin-Treated Mouse Lymphocytes (35592)

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Several reports on the interrelation between viruses and phytohemagglutinin (PHA)-stimulated lymphocytes have appeared. Most studies have utilized cultured human blood leukocytes. The present experiments were designed to determine whether PHA-stimulated mouse lymphocytes are capable of supporting the replication of viruses and to study the effect of viruses on the response of mouse lymphocytes to PHA. This system was chosen because of the potential advantages of an experimental animal model in the investigation on the role of the lymphocyte in viral infections. In addition, it offered the possibility of working with cells from genetically uniform individuals by employing an inbred mouse strain. To our knowledge no previous reports on the interaction between viruses and mouse lymphocytes have been published.

Materials and Methods. Preparation of lymphocyte cultures. Inbred 3- to 6-month-old mice of the C_{57}BL strain served as donors of lymphocytes. Mice were killed with chloroform. Cervical, axillary, brachial, inguinal, and mesenteric lymph nodes were excised aseptically and placed in Eagle's minimal essential medium. Lymph nodes of at least 6 animals were pooled. The lymph nodes were gently minced with scissors and the tissue fragments were screened through a stainless steel gauze into Eagle's medium. Pooled lymph nodes from 6 animals yielded, on the average, approximately 350×10^6 viable cells. Lymphocytes were separated from surface-adhesive cells by incubating the cell suspension in a bottle for 45–60 min at 37° followed by passage of the supernatant cells through a glass column (1) loosely packed with cotton wool. Cell viability was estimated with trypan blue dye and cell counts were obtained with a hemocytometer. Differential cell counts were made using Giemsa stain. Of the viable column purified cells, 96–98% were judged to be small and medium sized lymphocytes; and 2–4% large lymphocytes; no monocytes and macrophages were detectable. Lymphocytes were cultured at approximately 7.5×10^6 cells/ml, in aliquots of 1.5 ml/tube, in Eagle's minimal essential medium for suspension culture supplemented with 5% fetal calf serum. Culture tubes were incubated upright at 36° in a humidified atmosphere of 5% CO_2 in the air. Lyophilized phytohemagglutinin (PHA)-M(Difco) was dissolved in phosphate-buffered saline (5 ml/vial) and added at a final concentration of 0.3%. Lymphocyte stimulation was assessed by liquid scintillation counting of cellular incorporation of thymidine-^{3}H (2).

Viruses. Vesicular stomatitis virus (VSV) was the Indiana strain propagated and titered in L-cells (L-929) and contained 1.3×10^8 PFU/ml. Mengovirus (a large plaque mutant) was propagated and titered in L-cells and contained $10^{8.7}$ $TCID_{50}$/ml. Sendai virus was used in the form of infected chick embryo allantoic fluid. It was titered by a hemadsorption cell-counting method (3) in L-cells and contained 1.0×10^8 PFU/ml. Mouse adenovirus, obtained from Dr. W. P. Rowe of the National Institutes of Health, was propagated and titered in L-cells and contained $10^{6.5}$ $TCID_{50}$/ml. Reovirus type 2 (D5 Jones strain) and reovirus type 3 (Abney strain) were propagated and titered in primary rhesus monkey kidney cell cultures and contained, respectively, $10^{6.5}$ and $10^{8.0}$ $TCID_{50}$/ml. Herpes simplex virus (a strain isolated from the throat in this laboratory) was propagated and titered in a rabbit kidney cell line (T-17) established in the Laboratory of Virology, St. Elisabeth-Hospital, Til-

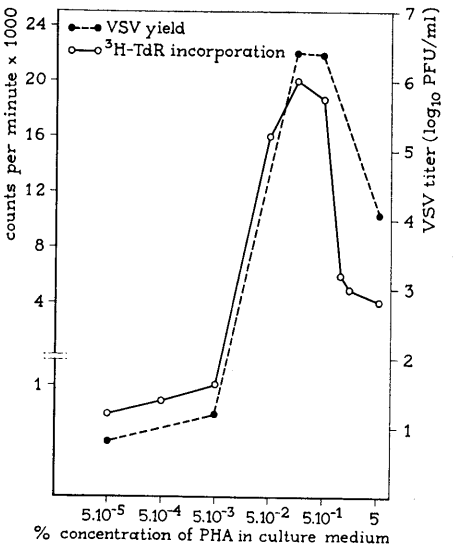

FIG. 1. Effect of PHA concentration on DNA synthesis and VSV replication in mouse lymphocyte cultures. Fresh PHA-treated cultures were inoculated with either control fluid or with 50 PFU of VSV/ml of culture. After 48-hr incubation, cultures were assayed for either DNA synthesis by determination of thymidine-^{3}H incorporation or for virus yield.

burg, The Netherlands. It contained $10^{6.5}$ $TCID_{50}$/ml.

Viral inoculation, viral assays. After 1 day of incubation, the medium of lymphocyte cultures was separated by centrifugation. The cellular pellets were resuspended in 1.5 ml of fluid consisting of 9 parts of fresh culture medium and one part of viral suspension. Cultures were incubated at 36°. Unadsorbed virus was not removed. At timed intervals after viral inoculation, culture fluid and cells from each of 2 or 3 tubes were collected and pooled. The pools were frozen and thawed rapidly 3 times to disrupt the cells. Infective virus was assayed by the plaque-counting method or the tube dilution method (see "Viruses"). Titrations by the tube dilution method employed 4 tubes/10-fold dilution, and the 50% end point ($TCID_{50}$) was calculated by the method of Kärber (4).

Results. Response of mouse lymphocytes to PHA. Initial experiments were performed to determine the dose–response curve of PHA in mouse lymphocyte cultures. Cultures were stimulated with PHA when the cultures were prepared (day 0), and harvested on day 2 after an 8-hr pulse with thymidine-^{3}H. Results as expressed in counts per minute are shown in Fig. 1. A maximal response was observed at a PHA concentration of 0.3% (final conc), which was chosen for further experiments. Subsequently, the time curve of the response of mouse lymphocytes to PHA was determined. Cultures were stimulated with PHA (0.3%) on day 0. The pattern of DNA synthesis, as determined by thymidine-^{3}H incorporation, was examined daily starting on day 1. To test the effect of serum concentration, these experiments were conducted with 3 groups of cultures supplemented with, respectively, 5, 10, and 20% fetal calf serum. The results of these experiments are shown in Fig. 2. The peak of thymidine-^{3}H incorporation invariably occurred on day 2. The magnitude of the response was dependent on the serum concentration; the best response was obtained at 5% serum concentration. There was no significant difference in cell viability between cultures with different serum concentrations. After 2 days of culture, 50–60% of the cells originally present in the cultures were viable; after 6 days, 25–30%; and after 9 days still 20–25%.

Replication of VSV in mouse lymphocyte cultures. Three groups of mouse lymphocyte cultures were set up with PHA. After 1 day of incubation, the cultures were infected with VSV. Group A received $10^{1.5}$ PFU/ml of culture; group B, 10^3 PFU; and group C 10^5 PFU. At timed intervals, 2 or 3 cultures of each group were harvested and assayed for

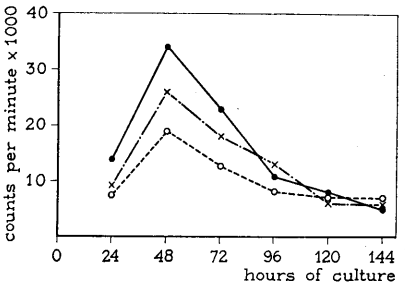

FIG. 2. Time course of the PHA response of mouse lymphocyte cultures containing 3 different concentrations of fetal calf serum: (●) 5% serum; (×) 10% serum; and (○) 20% serum.

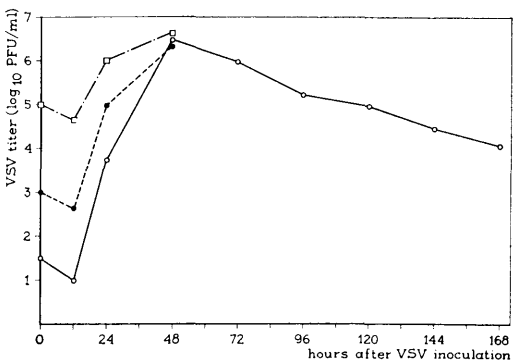

FIG. 3. Replication of VSV in PHA-treated mouse lymphocyte cultures inoculated with 3 different viral doses: (□) 10^5 PFU; (●) 10^4 PFU; and (○) $10^{4.5}$ PFU/ml of culture.

infective virus. An increase in viral titer was first detected at 24 hr after viral inoculation (Fig. 3). Maximal levels of virus (approx 5 × 10^6 PFU/ml of culture) were reached at 48 hr irrespective of the dose of the inoculum. To determine whether replication of VSV was dependent on PHA stimulation, lymphocyte cultures were established with decreasing dilutions of PHA. Five cultures were used per dilution. Two cultures were inoculated with 50 PFU of VSV/ml of culture simultaneously with the addition of PHA. After 48-hr incubation, the cultures were harvested and assayed for infective virus. The 3 remaining noninfected cultures were used for assay of DNA synthesis. The cultures were harvested at 48 hr after an 8-hr pulse with thymidine-^{3}H. As shown in Fig. 1, a direct relationship exists between virus yield and response to PHA, as measured by thymidine-^{3}H incorporation. Peak virus titers were reached at PHA concentrations which induced maximal DNA synthesis. There was no significant VSV replication in lymphocyte cultures treated with the lowest dose of PHA.

Replication of mengovirus in mouse lymphocyte cultures. Mouse lymphocyte cultures were set up with PHA. After 1 day of incubation, the cultures were infected with 10^3 $TCID_{50}$ of mengovirus/ml of culture. At timed intervals, 2 or 3 cultures were harvested and assayed for infective virus. Viral titers rose to slightly elevated levels after 48–72 hr, the log titer increase ranging from 0.5–0.75 in different experiments. Subsequently, a new group of PHA-treated mouse lymphocyte cultures was infected with 10^5 $TCID_{50}$ of mengovirus/ml of culture and serial passages of infected lymphocyte cultures were made. Cultures were harvested 72 hr after viral inoculation and 0.15 ml of harvest was passed to fresh cultures. There was an increase on each passage of the observed titer over the estimated titer based on dilution alone. The difference in log titer was found to be 5.0 by passage 5. These results indicate that PHA-stimulated mouse lymphocytes are capable of supporting the replication of mengovirus. This is further evident from experiments performed with a mengovirus strain which had been passaged 7 times in lymphocytes. The time course of replication of this viral strain in PHA-stimulated and nonstimulated mouse lymphocyte cultures was examined utilizing procedures similar to that just described. The cultures were infected with $10^{3.5}$ $TCID_{50}$ of mengovirus/ml of culture. In the absence of PHA, viral titers diminished with time (Fig. 4). In the presence of PHA, however, viral increase was noted. This increase proved to be greater than that obtained with the original nonpassaged viral strain (log viral titer increase of 2.0 versus 0.5–0.75).

Failure of replication of other viruses. In experiments similar to that described above, PHA-treated mouse lymphocyte cultures were tested for their capacity to support replication of other viruses. Cultures were inoc-

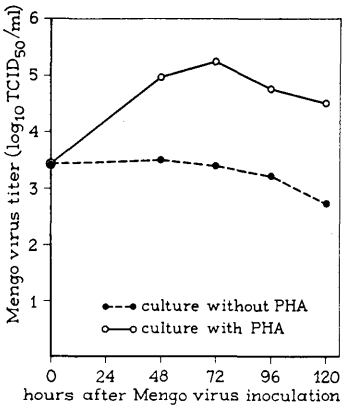

FIG. 4. Replication of mengovirus in PHA-treated and non-PHA-treated mouse lymphocyte cultures.

TABLE I. Effect of Viruses on PHA Response and Viability of Mouse Lymphocytes.

Virus[a]	Initial response (%)		Response at passage 5 (%)		Viral replication
	DNA synthesis[b]	Cell viability[c]	DNA synthesis	Cell viability	
VSV	7–29	39	18–24	41	Yes
Mengovirus	23–47	48	26–34	42	Yes
Herpes simplex virus	11–32	32	77–91	88	No
Sendai virus	87–96	89	95–103	97	No
Mouse adenovirus	95–98	91	93–99	102	No
Reovirus type 2	94–98	94	97–109	96	No
3	92–98	97	97–111	103	No

[a] PHA-treated cultures were inoculated with 0.15 ml of 1:10 diluted viral stock suspension or control fluid/culture. Five serial passages to fresh cultures were made.

[b] Lowest and highest value of thymidine-^{3}H incorporation in 3 experiments at 48 hr of culture. The values were related to the value in noninfected control cultures which was assigned as 100%.

[c] Mean value of 2 counts at 48 hr of culture. The values were related to the value in noninfected control cultures which was assigned as 100%.

ulated with 6×10^6 PFU of Sendai virus, $10^{4.0}$ $TCID_{50}$ of herpes simplex virus, $10^{4.5}$ $TCID_{50}$ of mouse adenovirus, $10^{5.0}$ $TCID_{50}$ of reovirus type 2 or $10^{5.0}$ $TCID_{50}$ of reovirus type 3/ml of culture. Serial passages of infected lymphocyte cultures were made 72 hr after viral inoculation. No evidence of viral replication was detected. Titers of Sendai virus and herpes simplex virus declined continuously from the first day after viral inoculation until no virus was recoverable after 4 days. Titers of mouse adenovirus and reovirus diminished at a slower rate. There was no significant difference on passage between the observed and the estimated viral titer based on dilution alone and these viruses failed to survive for more than 5 passages.

Effect of viruses on PHA response and viability of mouse lymphocytes. Mouse lymphocyte cultures were set up with PHA. The cultures were infected immediately with 0.15 ml of 1:10 diluted viral stock suspension (see "Materials and Methods") or control fluid/culture. Five cultures were used for each virus specimen or for the control fluid. All cultures were harvested after 2 days of incubation. Three cultures were used for assay of DNA synthesis. The 2 remaining cultures were used for cell viability counts, for assay of infective virus and for serial passage to fresh lymphocyte cultures. DNA synthesis of cells, cell viability, and virus yield were also determined after 5 serial passages. The results of the experiments are summarized in Table I. VSV, mengovirus, and herpes simplex virus inhibited the response of lymphocytes to PHA and caused a marked decrease in cell viability. Only VSV and mengovirus replicated sufficiently for the effect to be serially passaged. Slight inhibition of the PHA response and limited decrease in cell viability were found in cultures infected with Sendai virus. Mouse adenovirus, reovirus type 2 and reovirus type 3 failed to suppress the PHA response and to affect cell viability.

Discussion. Short-term cultures of lymph node lymphocytes have been established. Lymphocytes in PHA-treated cultures transformed and survived satisfactorily. The PHA response of these lymphocytes was similar to that observed with mouse blood lymphocytes (5). Of the viruses tested, VSV and mengovirus were capable of replicating in PHA-treated mouse lymphocytes. No significant viral replication occurred in the absence of PHA. These observations correspond with the results of studies on VSV replication in human lymphocyte cultures (6). The mechanism by which PHA induces viral replication in lymphocyte cultures remains to be elucidated. PHA induces a variety of biochemical changes in lymphocytes. In the present study, a maximal yield of VSV was

found at PHA concentrations which induced maximal stimulation of DNA synthesis, as measured by thymidine-^{3}H incorporation. However, VSV replication may be related to other metabolic changes. Viruses which failed to multiply in nonstimulated lymphocytes but replicated to high titers in stimulated lymphocytes have been shown to replicate as well in monocytes and macrophages (6–8) which are cell populations with a very low background of DNA synthesis. Furthermore, it was shown previously (6) that VSV replication in PHA-stimulated human lymphocytes takes place within 8 to 16 hr after viral inoculation. Since an increased synthesis of RNA and protein occurs within 2 to 10 hr after addition of PHA and DNA synthesis is increased only after 24 to 48 hr, it was suggested (6) that VSV replication might be associated with the earlier change in RNA or protein metabolism. Similarly, other studies have indicated that the period of maximal susceptibility of PHA-stimulated human lymphocytes to poliovirus infection coincides with the period of maximal RNA synthesis (9).

The finding that replication of mengovirus in mouse lymphocyte cultures was enhanced after serial passage in these cultures suggests the possibility of viral adaptation to growth in lymphocytes. Similar to this finding, Wallace (10) reported that Coxsackie A-15 virus became cytopathic for a strain of human lymphoblasts after 3 passages in these cell cultures. In contrast to observations with human lymphocytes (11), mouse lymphocytes failed to support the replication of herpes simplex virus. This might reflect variation in susceptibility between human and mouse lymphocytes. It is also conceivable that age-dependent resistance accounted for this failure. We used lymphocytes from 3-month-old, or older, mice. Previous studies (12) indicated that macrophages of 4-day-old suckling mice and adult mice can be infected with equal ease after inoculation with herpes simplex virus. However, suckling mouse macrophages infected other cells in contact with them, while infected adult macrophages did not.

The PHA response of mouse lymphocytes, as measured by thymidine-^{3}H incorporation, was inhibited by some but not all viruses tested, a finding similar to one previously reported for human lymphocytes (2). The decreased DNA synthesis could be accounted for by decreased cell survival. Our data suggest that the present system may prove useful as a model for further studies of the role of the lymphocyte in viral infections in mice.

Summary. PHA-stimulated mouse lymph node lymphocytes were capable of supporting the replication of VSV and mengovirus. Other viruses failed to replicate. The PHA response of the lymphocytes was inhibited by viruses which caused a decrease in cell viability.

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