BRIEF REPORT

Establishment of Long-term Lines from Small Aliquots of Normal Lymphocytes

By Susan W. Broder, P. R. Glade and K. Hirschhorn

LONG-TERM LYMPHOID CELL CULTURES have been established from peripheral blood from patients with a variety of benign and malignant lymphoproliferative disorders and from normal individuals.1,3 These cultures maintain a diploid chromosome number4 and appear to retain the genetic phenotype of the host,5 suggesting that they may provide a readily accessible source of cells for the study of human genetic variation. Whereas lymphoid suspension cultures have been established with relative ease from small aliquots of blood from patients with lymphoproliferative disorders,3 large quantities of blood have been required for the establishment of such cultures from normal individuals.3 If permanent lymphoid cell lines are to provide a useful source of cells for genetic study, the ability to establish such lines from any individual using small aliquots of peripheral blood must be developed.

Phytohemagglutinin (PHA), a phytomitogen derived from the kidney bean Phaseolus vulgaris, has the capacity to transform normal lymphocytes into blast-like cells associated with alterations in RNA and DNA synthesis followed by mitosis and cell division.6 It appears that normal human lymphocytes can be sustained in cell culture for longer periods of time in the presence of PHA than control cultures without PHA.7 The ability to survive for an extended period of time and proliferate in vitro are prerequisite conditions for the establishment of lymphoid cells in long term suspension culture. In the present studies, PHA has been utilized in an attempt to increase the potential of human blood lymphocytes for long term in vitro proliferation.

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MATERIALS AND METHODS

Lymphoid suspension cultures were prepared from the peripheral blood as previously described. As little as 10 ml of blood were withdrawn into a heparinized (Upjohn-100 units/ml whole blood) plastic syringe and allowed to sediment at 37°C. Leukocytes were obtained from the plasma supernatant by centrifugation at 250 x g for 30 minutes. The plasma was removed, the cells were counted, and replicate cultures containing 1 to 3 x 10⁶ cells were prepared in a total volume of 10 ml of the RPMI³ culture medium containing 20 per cent heat inactivated fetal calf serum and antibiotics in closed 30-ml plastic disposable flasks (Falcon Plastics). Cultures were incubated at 37°C and maintained by weekly gravity sedimentation of the cells, removal of 3 ml of supernatant fluid, and replenishment with fresh medium. Successful culture was manifested by a lowered pH of the culture medium, increase in cell numbers with clumping of cells in suspension, and the ability to pass the cell line continuously.

A highly purified, non-hemagglutinating PHA (Burroughs-Wellcome #E-119) was used throughout these investigations. This PHA has mitogenic activity at a dose of 0.025μg./ml, while its hemagglutinating activity is demonstrable only at 100-fold higher concentration (4.25 μg./ml.). The effect of this material on the doubling time of established lymphoid suspension cultures was studied on line PGIP-7 derived from the peripheral blood of a one-year-old child with primary herpes gingivostomatitis and maintained in long-term culture for six months. The effect of the minimum mitogenic dose of the PHA as well as several dilutions below this dose were studied.

RESULTS

Toxicity for an Established Lymphoid Cell Line

Direct cell counts by hemocytometer were conducted at intervals from 0 to 7 days. There was no apparent difference in repeated studies for each time interval observed between the numbers of cells present at each concentration of PHA from 0.025 μg./ml. to 0.00125 μg./ml. when compared to the non-PHA control. The cell population doubled in PHA and non-PHA control cultures every 24 to 48 hours. PHA #E-119, therefore, appeared to be nontoxic for established lymphoid cells and did not alter the replication of these cells.

The PHA was then tested for its toxicity to circulating precursor cells in the following experiment. Replicate cultures were prepared from a sample of peripheral blood of an individual with a viral gastroenteritis (Table 1). Two of the cultures received no PHA. Two samples were cultured with a single dose of 0.0025 μg./ml. PHA. This dose was a log₀ dilution below the minimum mitogenic dose. The fifth culture was incubated with a single aliquot of the minimum mitogenic dose of the PHA, i.e., 0.025 μg./ml. Four of these five samples became established in long-term culture by 49 days. No intact lymphoid cells were detectable in the fifth culture at this time, and it was discarded. The long term lines have now been in continuous culture for at

<table>
<thead>
<tr>
<th>Culture</th>
<th>Cell Count x 10⁶</th>
<th>PHA in μg./ml.</th>
<th>Days Until Established</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.3</td>
<td>No PHA</td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>2.3</td>
<td>No PHA</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>2.3</td>
<td>0.0025</td>
<td>Dead at 49</td>
</tr>
<tr>
<td>4</td>
<td>2.3</td>
<td>0.0025</td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td>2.3</td>
<td>0.025</td>
<td>49</td>
</tr>
</tbody>
</table>
least six months with passage twice weekly and show no signs of termination. The peripheral blood samples destined to establish in long-term suspension culture, therefore, did not appear to be adversely affected by addition of PHA at the beginning of culture. Similar results have been obtained from blood drawn from patients with infectious mononucleosis.

Since PHA #E-119, a non-hemagglutinating purified PHA, appeared to be non-toxic for lymphoid cells in long-term culture and the circulating precursor cells of such lines, this PHA was utilized in an attempt to increase the chance of establishing cultures from small aliquots of peripheral blood from normal donors. The cultures received either a single dose of PHA (concentrations of 0.025 μg/ml or 0.0025 μg/ml) or individual doses of these same concentrations weekly for four to ten weeks. Control cultures from the same donors received no PHA. Nine donors were studied in this manner. Long-term suspension cell lines were established from two of these donors in the peripheral blood cultures which had received PHA stimulation. None of the unstimulated cultures in these donors became permanent lymphoid cell lines. A series of 41 cultures attempted from small aliquots of peripheral blood from normal donors which received no PHA failed to yield a single long-term lymphoid cell line.

The nine normal donors in the present experiment include four donors whose cultures received a single initial dose of either 0.025 μg./ml or 0.0025 μg./ml PHA and five donors whose cultures received either 0.025 μg./ml or 0.0025 μg./ml PHA for four to ten weeks. The PHA-stimulated cultures of one donor in each of the two categories of stimulation became long-term lymphoid cell lines. The cultures which received no PHA failed to become permanent cell lines.

**DISCUSSION**

Primary lymphoid suspension cultures prepared from small aliquots of peripheral blood from normal donors have previously failed to become long-term lymphoid suspension cultures. Initial attempts to establish permanent lymphoid suspension cultures from normal individuals required a two-volume plasmapheresis and resulted in an approximately 30 per cent success rate. This success rate has recently been improved to at least 75 per cent. This method, however, requires multiple simultaneous cultures with frequent pooling of cells to maintain optimal concentrations. It is time consuming, expensive, and impractical for general use in pediatric populations.

Previously reported attempts to establish lymphoid cell lines by PHA stimulation had resulted in failure. This failure may be attributed to the use of crude PHA products which may contain toxic factors. Stimulation of cultures prepared from approximately 10 ml. of blood from normal individuals using PHA #E-119 has increased the number of successful cultures of this type to about 20 per cent. Careful study of the culture conditions and the kinetics of the PHA stimulated culture system should improve the success rate and may make this a simple and predictable method for the establishment of long term lymphoid suspension cultures from normal individuals.

Initial studies suggest that long term lymphoid cell cultures may be useful in in vitro models for the study of human genetic variation. Histocompatibility
testing for human leukocyte antigens (HLA) has shown that the cells in long
term lymphoid suspension culture retain the HLA specificity of the donor. Similarly, the persistence of host enzymes in long term lines can be demonstrated utilizing genetically polymorphic enzyme markers. Cell lines from normal individuals and patients with defined metabolic defects should permit the purification of normal and defective enzymes for comparative study.

SUMMARY

A highly purified, non-hemagglutinating PHA used in minimal blastogenic
doses was non-toxic to established human lymphoid cell lines. PHA given at
the beginning of primary culture or repeatedly throughout the initial six
weeks of culture did not interfere with the establishment of peripheral lympho-
cytes in long-term suspension culture. Permanent cell lines have been obtained
following weekly administration of PHA in blastogenic and subthreshold doses. Nonhemagglutinating PHA appeared to be non-toxic to these peripheral lymphocyte cultures and may increase their potential for long-term proliferation.

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