The Effects of Varying Concentrations of Phytohemagglutinin and Conditions of Incubation on the Enhanced Survival of Human Peripheral Lymphocytes in Vitro

By Maxwell Richter and Charles K. Naspitz

In a previous communication, it was reported that normal human peripheral lymphocytes could be sustained in a viable state in cell culture for long periods of time (forty-two days) in the presence of phytohemagglutinin (PHA) whereas control cultures in the absence of PHA survived for a much shorter period of time. The concentration of PHA used was one which did not induce blastogenesis of lymphocytes in vitro and was referred to as "subthreshold PHA." Only one concentration of PHA was used. In the present study, the role of different concentrations of PHA, the volume of the cell culture, and the effect of changing the culture medium at regular intervals of time during the incubation period were investigated as to their effects on the survival of the lymphocyte in vitro.

Materials and Methods

Phytohemagglutinin-M (PHA) (Difco Laboratories, Detroit, Michigan) was used throughout. The contents of a vial were diluted with 5 ml. of Medium 199 (referred to as "undiluted PHA") and then further diluted one hundred-fold with Medium 199.

Heparinized blood was obtained from normal, healthy volunteers by bleeding from the antecubital vein (100 units heparin per ml. of blood). The blood was pipetted into sterile plastic tubes and maintained at 37 C. to allow the red cells to settle. At the appropriate time, the leukocyte-rich plasma layer was removed and the white cell count performed by hemocytometer. The white cells were diluted in Medium 199 (Microbiological Products, Bethesda, Maryland, U.S.A.), the final suspension containing 15 per cent autologous plasma, 100 units penicillin, and 100 μg streptomycin per ml. The cell concentration was 10⁶ per ml. Four ml. of the cell suspension were pipetted into sterile plastic tubes (Falcon), and where specified PHA, diluted one hundred-fold, was added in a volume of 0.10, 0.30, or 0.60 ml. The tubes were tightly sealed and maintained at 37 C. Where specified, the medium, containing PHA, was changed every seven days without centrifugation using either homologous or autologous serum at a concentration of 15 to 20 per cent.

Three days before termination of culture (days 18, 32, and 46), 0.25 ml. of PHA diluted...
tenfold was added to the tubes (suprathreshold concentration of PHA). Twenty-four hours before termination of culture, 2 µc. tritiated thymidine and 1 µg. carrier thymidine in 0.1 ml. were added to each tube. At the conclusion of culture, 100 µg. nonradioactive thymidine were added to the tubes and the latter were centrifuged at 1,000 rpm for 10 min. The contents of several of the tubes were then washed twice with Medium 199, resuspended in a volume less than 0.1 ml., and smears made on gelatin-coated slides and cover slips which were heat-fixed at 60 to 70 C. by means of a hair dryer. The slides were analyzed by radioautography (see below). The cover slips were stained with Jenner-Giemsa. The remaining tubes were washed twice with 2 ml. volumes of 5 per cent trichloracetic acid. One half ml. of Hyamine (Packard Instruments Co.) was added to each tube and the tubes were allowed to undergo digestion at room temperature, in the dark, for twenty-four hours. The contents of each tube were then transferred to a scintillation counting vial with 2 aliquots of absolute ethanol (0.6 ml. total) and maintained in a 70 C. water bath for one hour. Fifteen ml. of scintillation solution (400 Gm. naphthalene, 28 Gm. PPO, and 1.2 Gm. POPOP, made up to 3,800 ml. with dioxane) were added to each vial and the vials counted in a Packard Model 4000 Scintillation Counter.

For radioautographic examination, the slides were coated with Kodak Liquid Nuclear Track Emulsion, type NTB-2, stored at 4 C. for two to four days and developed and stained with Jenner-Giemsa.

The phagocytic capacity of the cell suspensions during the culture was tested by adding a drop of a 10 per cent suspension of India Ink two hours prior to the termination of culture. The suspensions were washed twice with Medium 199, and smears made on gelatin-coated slides were examined either by phase-contrast microscopy or stained with Jenner-Giemsa and examined by light microscopy.

**RESULTS AND DISCUSSION**

The results of representative experiments are illustrated in Table 1. When the medium was changed at weekly intervals, it was observed that 0.1 or 0.3 ml. of PHA was superior in maintaining the lymphocytes in vitro as compared to 0.6 ml. PHA during the entire period of culture. Whether the medium was changed or not did not appreciably affect the cultures incubated with subthreshold PHA during the first thirty-five days. Analysis of the cultures subsequent to this time leaves no doubt as to the benefit of changing the medium every seven days since these latter cultures incorporated two to eight times the amount of radioactive thymidine as did cultures in which the media were not changed during the forty-nine day period of culture. Furthermore, although the volumes in the culture tubes were increased weekly in those in which medium was not changed during the culture period due to the weekly addition of "subthreshold PHA," the incorporation of tritiated thymidine by these cells did not appear to be affected.

One of the cultures was maintained up to fifty-nine days. However, it appeared to deteriorate markedly between days 46 and 56 since the incorporation of tritiated thymidine during the day 56 to 59 period was negligible.

Large numbers of labeled blast cells were observed in the cultures at days 21, 35 and 49 (Fig. 1). Most conspicuous were the large number of cell aggregates which were observed in the cultures which were incubated in the presence of subthreshold PHA. These aggregates or rosettes (Fig. 2) were especially frequent in the long-term cultures subsequent to the addition of the suprathreshold PHA (i.e., days 21, 35, 49). They invariably consisted of a large centrally located macrophage to which adhered a large number of blast cells...
Table 1.—Effect of Varying Subthreshold Concentrations of Phytohemagglutinin-M and Weekly Changes of the Culture Medium on the Survival of Human Peripheral Lymphocytes in Vitro

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Medium Containing</th>
<th>Medium Changed Weekly</th>
<th>Uptake of Tritiated Thymidine at Day (\times 10^3)×</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3⁵</td>
</tr>
<tr>
<td>1.</td>
<td>No PHA</td>
<td>Yes</td>
<td>92 (.25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.1 ml. PHA</td>
<td>Yes</td>
<td>80 (.50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.3 ml. PHA</td>
<td>Yes</td>
<td>190 (.28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.6 ml. PHA</td>
<td>Yes</td>
<td>176 (.25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td>2.</td>
<td>No PHA</td>
<td>Yes</td>
<td>106 (.28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.1 ml. PHA</td>
<td>Yes</td>
<td>50 (.12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.3 ml. PHA</td>
<td>Yes</td>
<td>68 (.11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.6 ml. PHA</td>
<td>Yes</td>
<td>86 (.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td>3.</td>
<td>No PHA</td>
<td>Yes</td>
<td>156 (.78)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.1 ml. PHA</td>
<td>Yes</td>
<td>25 (.58)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.3 ml. PHA</td>
<td>Yes</td>
<td>75 (.32)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.6 ml. PHA</td>
<td>Yes</td>
<td>110 (.78)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td>4.</td>
<td>No PHA</td>
<td>Yes</td>
<td>130 (.42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.1 ml. PHA</td>
<td>Yes</td>
<td>130 (2.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
<td>—</td>
</tr>
</tbody>
</table>

a Average of triplicate determinations, given as counts per minute.
b Suprathereshold PHA (0.25 cc., 1:10) added on day 0.
c Suprathereshold PHA (0.25 cc., 1:10) added on day 18.
d Suprathereshold PHA (0.25 cc., 1:10) added on day 32.
e Suprathereshold PHA (0.25 cc., 1:10) added on day 46.
f The values within the brackets represent the uptake of tritiated thymidine in the absence of additional suprathereshold concentration of PHA.

as well as several unlabeled small and medium-sized lymphocytes. Whether this type of cell-cell interaction represents a transfer of information from one cell to the other, as has been previously suggested by several authors, or whether it is an innocuous relationship induced by the condition in the tissue culture medium cannot be ascertained. Furthermore, whether the macrophage seen at day 49 of culture is an original survivor in the culture or a transformed lymphocytoid cell, a possibility which has previously been suggested by Robbins and Elves et al., is not certain.

Many macrophages were seen with carbon particles in their cytoplasm at all times during the culture, thus attesting to the phagocytic capacity of these
The central macrophage observed in the cell aggregates referred to above were not always phagocytic, however.

The amount of PHA originally used to maintain the lymphocytes in vitro, referred to as subthreshold PHA, was 0.1 ml. of a 1:100 dilution of the original PHA solution. This concentration of PHA does not induce thymidine incorporation when incubated with the fresh leukocyte suspension during the initial three days of culture. The addition of either 0.3 or 0.6 ml. of PHA diluted 1:100, was as capable of maintaining a viable population of cells as was 0.1 ml. of the PHA solution, at least up to day 35 of culture. These quantities (0.3 and 0.6 ml.) of PHA were certainly capable of initiating blastogenesis and thymidine.
Table 2.—In Vitro Incorporation of Tritiated Thymidine by Human Peripheral Lymphocytes Incubated with Varying Concentrations of PHA-M for 72 Hours

<table>
<thead>
<tr>
<th>Quantity of PHA Added</th>
<th>Uptake of Tritiated Thymidine ($\times 10^4$)* by Cells of Individual</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 ml. undiluted</td>
<td>105 110 150 140</td>
</tr>
<tr>
<td>0.25 ml. diluted 5-fold</td>
<td>145 220 190 182</td>
</tr>
<tr>
<td>0.25 ml. diluted 20-fold</td>
<td>115 165 220 220</td>
</tr>
<tr>
<td>0.25 ml. diluted 80-fold</td>
<td>10 25 15 3</td>
</tr>
<tr>
<td>0.25 ml. diluted 100-fold</td>
<td>2 5 3 2</td>
</tr>
<tr>
<td>0.1 ml. diluted 100-fold</td>
<td>1 0.5 0.8 0.3</td>
</tr>
<tr>
<td>0.3 ml. diluted 100-fold</td>
<td>10 23 16 5</td>
</tr>
<tr>
<td>0.6 ml. diluted 100-fold</td>
<td>30 80 76 25</td>
</tr>
</tbody>
</table>

*Each value represents the mean of triplicate determinations. Expressed as counts per minute.

dine incorporation during the first three days of culture to a degree 10 to 40 per cent of cultures incubated with the optimal suprathreshold concentration of PHA (Table 2). At day 35, the uptake of tritiated thymidine by the cultures containing 0.3 to 0.6 ml. of PHA was similar to those maintained in the medium containing only 0.1 ml. PHA during the entire culture period (Table 1). One may surmise that the lymphocytes which underwent transformation during the initial period of culture with 0.3 and 0.6 ml. PHA probably did not survive by day 35 in the cultures containing 0.1 ml. of PHA, or else the incorporation of tritiated thymidine in the latter culture would have been greater than in the former cultures. It is obvious, therefore, that lymphocytes with different degrees of sensitivity to PHA exist in the original population of cells. Whether any relationship exists between the "short-lived" and "long-lived" lymphocytes and the relative sensitivity to PHA remains to be determined. The authors feel that more fruitful results will be obtained using pure suspensions of lymphocytes at the beginning of culture rather than whole buffy coat.

SUMMARY

1. Various concentrations of PHA were utilized to determine the optimal subthreshold concentration of PHA for the maintenance of lymphocytes in vitro. Although 0.1, 0.3 and 0.6 ml. of PHA (diluted one hundred-fold) appeared to be similar in effectiveness during the initial 35 days of culture, 0.1 or 0.3 ml. of PHA were more effective over longer periods of time.

2. Whether the culture medium was changed at weekly intervals or not had little effect on the culture during the initial 35 days. Over the long run, however, changing the culture medium every week resulted in a larger number of surviving lymphocytes.

3. Many phagocytic cells could be observed during the entire period of culture. Most conspicuous were clumps of cells consisting of a central macrophage surrounded by blasts and a few small and medium-sized lymphocytes.
SUMMARIO IN INTERLINGUA

1. Varie concentrationes de PHA eseva utilisate pro determinar le optime concentration subliminal de PHA pro le mantenentia de lymphocytos in vitro. Ben que 0,1, 0,3, e 0,6 ml de PHA (dilutionate centuplicamente) pareva esser simile in efficacia durante le prime 35 dies del culturation, pro plus longe periodos 0,1 e 0,3 ml de PHA eseva plus efficace.

2. So o non le medio de culturation eseva excambiate a intervallos septimanal habeva pauc influentia super le culturas durante le prime 35 dies. A plus longe terminos, del altere latere, le excambio septimanal del medio de cultura resultava in plus alte numeros de lymphocytos supervivente.

3. Multe cellulas phagocytic poteva esser observate durante le integre periodo del culturation. Le constatation le plus conspicue esseva aggregatos de cellulas consistente de tin macrophago central que esseva circumvallate de blastos e un basse numero de micre e intermediari lymphocytos.

ACKNOWLEDGMENT

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REFERENCES


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