LYMPHOCYTE TRANSFORMATION INDUCED BY AUTOLOGOUS CELLS

II. Stimulation by Mitogen-Induced Lymphoblasts*

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The stimulation of lymphocyte transformation by autologous human lymphoblasts established in continuous culture has been reported by several laboratories (1–6). The nature of the determinants on cultured lymphoblasts that stimulate transformation of autologous lymphocytes is not clear, but may be related to what appears to be a universal infection (latent or expressed) of all lymphoblast lines by the Epstein Barr (EB)[1] virus (7–10).

The aim of the present study was to investigate the capacity of lymphoblasts in which EB virus infection is not expressed to stimulate autologous lymphocyte transformation. The following report shows that lymphoblasts that result from the incubation of blood lymphocytes with phytohemagglutinin (PHA) or concanavalin A (Con A) stimulate the transformation of autologous lymphocytes. The stimulatory determinants that appear on mitogen-induced lymphoblasts may be related to those determinants on lymphoblast lines or leukemic leukocytes that stimulate autologous lymphocyte transformation.

Materials and Methods

Heparin used in these studies was obtained from Organon, Inc., West Orange, N. J. Medium RPMI 1640 with penicillin 100 U/ml and streptomycin 50 μg/ml, to which 2 μmol glutamine/ml were added immediately before use, was purchased from Associated Biomedic Systems, Inc., Buffalo, N. Y. Cultures were placed in sterile, plastic, 5-ml test tubes produced by Falcon Plastics, Oxnard, Calif. Concanavalin A, fetuin, and methyl alpha mannoside were purchased from the Sigma Chemical Co., St. Louis, Mo. Phytohemagglutinin-P was obtained from Difco Labs., Detroit, Mich. Tritiated thymidine (specific activity 6.7 Ci/mM) and toluene-Liquifluor scintillant were purchased from the New England Nuclear Corp., Boston, Mass. NCS solubilizer was purchased from Nuclear-Chicago, Des Plaines, Ill.

20–35 ml of heparinized blood (10 U/ml) were sedimented in the syringe at 37°C for 2 h. The white cell-rich plasma was expressed and the lymphocyte count adjusted to 2×10^6 lymphocytes/ml plasma. Replicate 2-ml lymphocyte cultures were established in 5-ml plastic

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; EB, Epstein Barr; PHA, phytohemagglutinin.

test tubes and consisted of 0.4 ml of white cell-rich plasma containing 8×10^5 lymphocytes and 1.6 ml of culture medium. Where indicated, 80 μg of Con A or 1.5 μl of PHA-P stock solution (made up by adding 5 ml of sterile water to one vial of PHA-P) were added and the cultures incubated at 37°C in a 5% CO_2/95% air environment for 66 h. To measure lymphocyte transformation 2.5 μCi of [^{3}H]thymidine in 25 μl was added to each culture and the incubation continued for 6 h. Replicate cultures incubated in the presence or absence of mitogen were used to stimulate autologous lymphocyte transformation. PHA or Con A was added to cultures not previously exposed to mitogen. 1 h later cells from all cultures were collected by centrifugation (400 *g* for 10 min), washed twice with culture medium, and resuspended in medium containing either 5 mg fetuin/ml medium (cultures exposed to PHA) or 0.05 M methyl alpha mannoside (cultures exposed to Con A) and incubated for 2 h at 37°C. The cells were again collected by centrifugation, washed twice with medium, and resuspended in 1.6 ml of medium. All stimulating cells were irradiated with 4500 R from a cesium 137 source. 8×10^5 responding autologous lymphocytes were added in 0.4 ml of autologous plasma and incubated 5 days at 37°C in a 5% CO_2/95% air environment. Lymphocyte transformation induced by autologous lymphoid cells was measured by the incorporation of thymidine as described above. After the 6 h incubation with tritiated thymidine, the culture tubes were filled with 0.15 M sodium chloride and the cells collected by centrifugation. The supernatant was discarded and a drop of diluted human serum added to each tube as carrier protein. 5% trichloroacetic acid was added and the resulting precipitate was collected by centrifugation and washed once with trichloroacetic acid. The precipitate was dissolved in 1 ml of NCS solubilizer, and 0.6 ml of solubilizer was added to 10 ml of toluene-Liquifluor scintillant and counted in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Removal of Active Mitogen from Mitogen-Induced Lymphoblasts.—Lymphocytes incubated with PHA or Con A are transformed into lymphoblasts in 72 h. Washed, irradiated, mitogen-induced lymphoblasts stimulate autologous lymphocyte transformation more than do leukocytes exposed to mitogen for 1 h (Table I). However, the marked stimulation of lymphocyte transformation by mitogen-exposed leukocytes suggests that incomplete removal of active mitogen, despite repeated cell washing, is largely responsible for the stimulation of autologous lymphocyte transformation.

Mitogen bound to leukocytes was dissociated by incubation of mitogen-exposed cells with inhibitors of mitogen action. These compounds presumably compete with lymphocyte surface receptors for mitogen and thus dissociate mitogen bound to cell surface sites. Leukocytes exposed to PHA were washed and incubated with culture medium containing 5 mg fetulin/ml of culture medium; leukocytes exposed to Con A were incubated with culture medium containing 0.05 M methyl alpha mannoside. After incubation with mitogen ligands, the cells were washed twice, irradiated, and mixed with fresh autologous lymphocytes. Under these conditions the capacity of mitogen-exposed leukocytes to stimulate lymphocyte transformation was reduced more than 90%. Nonetheless, this preparation still stimulated more lymphocyte transformation than did lymphocytes not exposed to mitogen (Table I). This type of preparation clearly showed the capacity of mitogen-induced lymphoblasts to stimulate autologous lymphocyte transformation (Table II). Cultures containing only

TABLE I

*Removal of Mitogen from Leukocytes Exposed to Phytohemagglutinin or Concanavalin A**

Preparation of stimulator cells (additions to culture)		Transformation of autologous lymphocytes‡ (cpm thymidine incorporated/culture × 10^{-3})
Day 0	Day 3	
PHA	None (3)§	43.4 (22.6–53.6)
None	PHA (3)	39.7 (30.6–42.2)
Con A	None (3)	33.2 (15.6–44.1)
None	Con A (3)	28.3 (21.4–32.6)
None	PHA and fetuin (8)	2.2 (0.2–12.9)
None	Con A and mannoside (5)	1.0 (0.7–1.8)
None	None (6)	0.5 (0.2–0.9)

* 8×10^5 lymphocytes were placed into culture on day 0. PHA or Con A was added to cultures at the inception of the lymphocyte culture (day 0) or 66 h later (day 3). 1 h later the cells were collected by centrifugation and the leukocytes washed three times with culture medium, resuspended in 1.6 ml of culture medium, irradiated, and added to 0.4 ml of autologous plasma containing 8×10^5 fresh autologous lymphocytes. Where indicated the mitogen-exposed lymphocytes were washed and incubated with mitogen ligands for 2 h at 37°C after which they were washed three times with medium. The amount of thymidine incorporated into lymphocyte DNA was measured on day 8. Irradiated cultures to which no fresh cells were added incorporated less than 500 cpm/culture.

‡ Results presented include mean and range of results, given in parentheses.

§ Number of experiments performed.

TABLE II

*Stimulation of Lymphocyte Transformation by Autologous Mitogen-Induced Lymphoblasts**

Stimulating cells	Transformation of autologous lymphocytes‡ (cpm thymidine incorporated/culture × 10^{-3})	
Leukocytes exposed to Con A (3)§	1.0	(0.7–1.2)
Lymphoblasts induced by Con A (3)	8.1	(4.3–12.6)
Leukocytes exposed to PHA (9)	2.1	(0.2–12.9)
Lymphoblasts induced by PHA (9)	17.5	(0.5–30.3)
Leukocytes not exposed to mitogen (3)	0.8	(0.5–0.9)

* 8×10^5 lymphocytes were incubated in culture medium containing 20% autologous plasma for 66 h in the presence or absence of Con A 40 μg/ml or PHA-P 0.67 μl/ml. At 66 h mitogen was added to those cultures not previously exposed to mitogen. 1 h later cells were collected by centrifugation. All leukocytes incubated with PHA were resuspended in 1 ml of medium containing 5 mg of fetuin for 2 h at 37°C; all leukocytes incubated with Con A were resuspended with medium containing 0.05 M methyl alpha mannoside. All leukocytes were then collected by centrifugation, washed twice with medium and resuspended in 1.6 ml of medium, irradiated (4500 R), and mixed with 0.4 ml of autologous plasma containing 8×10^5 lymphocytes. 5 days later the incorporation of tritiated thymidine by each culture was measured.

‡ Results presented include mean and range of results in parentheses.

§ Number of experiments performed.

irradiated stimulator cells or irradiated stimulatory cells never exposed to mitogen and fresh autologous lymphocytes incorporated less than 900 cpm.

Stimulation of Lymphocyte Transformation by Autologous Mitogen-Induced Lymphoblasts.—The capacity of mitogen-induced lymphoblasts to stimulate autologous lymphocyte transformation is clearly demonstrated if mitogen ligands are used to dissociate mitogen bound to leukocytes (Table II). Although the "background" stimulation of thymidine incorporation into lymphocyte DNA by mitogen-exposed leukocytes was highly variable, it generally exceeded that observed when irradiated leukocytes never exposed to mitogen were mixed with autologous lymphocytes. Mitogen-induced lymphoblasts stimulated autologous lymphocyte transformation in each of six normal subjects tested. Previously we found that lymphoblasts in continuous culture from each of these persons stimulated autologous lymphocyte transformation. In three experiments Con A-induced lymphoblasts stimulated 4, 6, and 18 times more thymidine incorporation by autologous lymphocytes than did Con A-exposed lymphocytes. In nine experiments performed with PHA, eight showed that PHA-induced lymphoblasts stimulated 2.5–40 times more thymidine incorporation by autologous lymphocytes than did PHA-exposed leukocytes. Included in the data presented in Table II is a single experiment in which no stimulation by PHA-induced lymphoblasts was observed. This subject was restudied and PHA-induced lymphoblasts were found to stimulate autologous lymphocyte transformation.

Kinetics of Lymphocytes Transformation Induced by PHA and by Autologous PHA-Induced Lymphoblasts.—Lymphocytes incubated with PHA are stimulated to incorporate thymidine in association with morphological transformation. The peak rate of thymidine incorporation into lymphocyte DNA occurs 3–4 days after exposure to PHA. In contrast, the peak rate of thymidine incorporation after mixture of two allogeneic lymphoid populations occurs after 5–6 days. Thymidine incorporation into lymphocyte DNA was studied at various times after exposure of lymphocytes to PHA or to PHA-induced autologous lymphoblasts (Fig. 1). Thymidine incorporation by lymphocytes incubated with PHA reaches a sharp maximum after 3 days with a rapid decline thereafter. In contrast thymidine incorporation stimulated by PHA-induced lymphoblasts was maximal 5 days after the initiation of mixed leukocyte culture, and this preparation retained the capacity to stimulate thymidine incorporation for as long as 9 days. The different time-course of thymidine incorporation by PHA and PHA-induced lymphoblasts supports the conclusion that lymphocyte transformation stimulated by PHA-induced lymphoblasts is not attributable to the incomplete removal of active PHA.

Appearance of Determinants on PHA-Induced Lymphoblasts that Stimulate Autologous Lymphocyte Transformation.—The incorporation of thymidine by lymphocytes incubated with PHA is not significantly increased for 36–48 h. There is then a rapid increase in the incorporation of thymidine into lymphocyte

DNA that is maximal at 72–96 h. At this time the majority of lymphocytes in culture have transformed into lymphoblasts. The capacity of lymphocytes incubated with PHA for various periods of time to stimulate autologous lymphocyte transformation was studied in order to correlate the appearance of stimulatory activity with morphological transformation. The results of five experiments in which lymphocytes were incubated with PHA for 24–72 h before their mixture with autologous lymphocytes are summarized in Table III. The results are expressed relative to the stimulation of thymidine in-

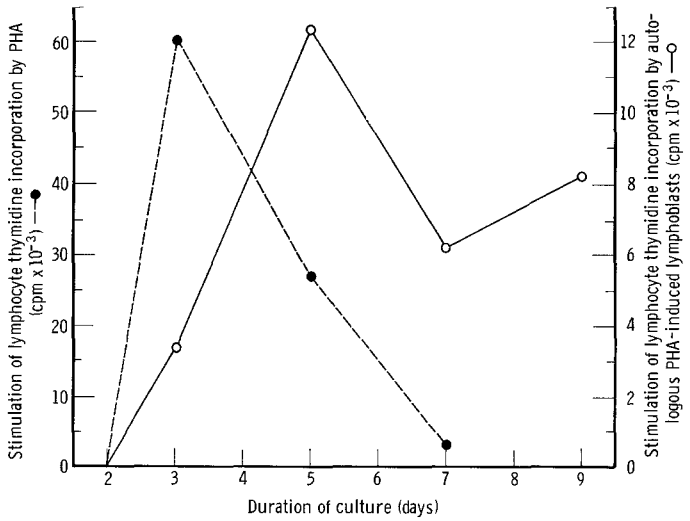

FIG. 1. Kinetics of lymphocyte transformation induced by PHA and by autologous PHA-induced lymphoblasts. Lymphocytes were incubated with PHA or with irradiated PHA-induced lymphoblasts for 2–9 days. The PHA-induced lymphoblasts were produced by the incubation of lymphocytes with PHA for 72 h. PHA was removed from the lymphoblast preparation by washing and incubation with 5 mg fetuin/ml medium. Lymphocyte transformation was measured by the incorporation of tritiated thymidine into lymphocyte DNA.

corporation by leukocytes exposed to PHA for 1 h. In two experiments, lymphocytes incubated with PHA for 24 h produced maximal stimulation of autologous lymphocyte transformation. In two experiments maximal stimulatory ability appeared after a 48 h incubation with PHA and in one experiment maximal stimulation was seen after 72 h. Clearly, the capacity of lymphocytes exposed to PHA to stimulate lymphocyte transformation does not develop in parallel with morphological transformation. Thus, when results from each experiment are expressed as a percentage of the mean normalized maximal stimulation of lymphocyte transformation, the most stimulatory lymphocyte preparation appears after a 24–48 h incubation with PHA although the maximal concentration of lymphoblasts appears after 72 h of incubation.

TABLE III

Capacity of Leukocytes Incubated with Phytohemagglutinin to Stimulate Autologous Lymphocyte Transformation

Experiments	Mixed leukocyte reaction using autologous leukocytes incubated with PHA for various times*			
	hours			
	1	24	48	72
1	1.0‡	2.3	33.3	13.7
2	1.0	N.D.	12.7	14.0
3	1.0	4.2	N.D.	1.0
4	1.0	6.8	N.D.	3.1
5	1.0	N.D.	3.1	2.6
Mean of normalized results§	—	70%	90%	48%

* Replicate cultures of leukocytes were established on day 0. PHA was added to cultures at the initiation of the culture 24, 48, or 66 h later. Cells from all cultures were collected by centrifugation at 67 h, washed once with medium and resuspended in medium containing 5 mg fetuin/ml medium, and incubated for 2 h at 37°C. Cells from each culture were washed twice with medium, resuspended in 1.6 ml of medium, irradiated, and mixed with 8×10^5 autologous lymphocytes in 0.4 ml of autologous plasma. The mixed cultures were incubated for 120 h. The amount of tritiated thymidine incorporated into lymphocyte DNA was assayed during a 6 h pulse.

‡ Results expressed as the fraction: counts per minute stimulated by leukocytes incubated with PHA for various times/counts per minute incorporated by cultures stimulated by leukocytes incubated with PHA for 1 h. The figure underlined is the maximal stimulation for that experiment.

§ Each experiment was normalized by expressing the values after exposure to PHA for different time periods as a percent of the peak response which was taken as 100%. The normalized results of the five experiments were averaged and the percent of peak after leukocyte incubation with PHA for 24, 48, or 72 h is presented.

DISCUSSION

Lymphoblasts grown in continuous culture stimulate the transformation of autologous lymphocytes (1–6). The nature of the stimulatory determinants on these cells is not certain. As all lymphoblast lines carry overt or latent infection with EB virus (7–10), the contribution of viral antigens to the stimulation of lymphocyte transformation must be considered. Lymphoblast lines that have no demonstrable EB viral antigens stimulate autologous lymphocyte transformation (5, 11). On the other hand, EB viral antigens themselves stimulate the transformation of lymphocytes obtained from persons with serological evidence of previous infection with the EB virus (12).

The participation of EB viral antigens in the stimulation of lymphocyte transformation by lymphoblasts was explored by examining the capacity of lymphoblasts not expressing the EB virus to stimulate autologous lymphocyte

transformation. Lymphoblasts that result from the incubation of lymphocytes with PHA have not been found to express EB virus (3, 13). Flier et al. (3) and Hardy, Knight, and Ling (11) have suggested that PHA-induced lymphoblasts stimulate the transformation of autologous lymphocytes.

The present studies demonstrate that lymphocytes incubated with PHA or Con A for 24–72 h develop the capacity to stimulate autologous lymphocyte transformation. This stimulation of lymphocyte transformation does not depend upon the incomplete removal of active mitogen from the stimulating cell preparation. In fact, as more complete dissociation of mitogen bound to the leukocyte preparation is effected by mitogen ligands, the stimulation of lymphocyte transformation by these cells becomes more apparent. Further the kinetics of lymphocyte transformation stimulated by PHA-induced lymphoblasts is different from that stimulated by PHA. The capacity of lymphocytes incubated with PHA to stimulate autologous lymphocyte transformation is maximal after 24–48 h, i.e., before the burst of thymidine incorporation and morphological transformation that occurs after 72–96 h of incubation of lymphocytes with PHA. This suggests that cell division need not precede the development of the stimulatory determinants.

These results suggest the appearance of a "new" determinant on normal lymphocytes incubated with mitogen. Fox, Sheppard, and Burger (14) showed that normal cells during restricted periods of their cell cycle display surface determinants that are continuously present on polyoma virus-transformed murine cells. Whether the determinants that appear on normal lymphocytes stimulated to cell division by mitogen, which are recognized as "foreign" by autologous lymphocytes, are related to tumor-specific determinants found on leukemic leukocytes remains the subject for further study.

SUMMARY

Lymphocytes incubated with phytohemagglutinin or concanavalin A develop the capacity to stimulate autologous lymphocyte transformation. This is not attributable to residual mitogen contaminating the lymphoblastic cell preparation as: (*a*) the dissociation of mitogen from the lymphoblastic cell preparation increases the degree of stimulation observed and (*b*) the kinetics of lymphocyte transformation stimulated by phytohemagglutinin-induced lymphoblasts is different from that stimulated by phytohemagglutinin. The appearance of the stimulatory determinants on lymphocytes exposed to phytohemagglutinin precedes morphological transformation.

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