Studies of the Delayed Response of Phytohemagglutinin (PHA) Stimulated Lymphocytes in 25 Chronic Lymphatic Leukemia Patients Before and During Therapy

By Bertha A. Bouroncle, Kathryn P. Clausen and Joan F. Aschenbrand

The capability of lymphocytes to transform in vitro into "blastoid" cells, under the effects of stimulation by phytohemagglutinin (PHA) in cultures, is currently utilized as one of the most popular in vitro technics for the study of some aspects of lymphocyte function.

Investigators have reported contradictory results in the response of lymphocytes in chronic lymphatic leukemia (CLL) when challenged with PHA. Earlier reports considered it to be normal,1 while others reported it to be either largely absent2-4 or markedly decreased.5-6 A delayed response has been reported12 more recently.

The present report is a long-term follow-up study of the transformation of lymphocytes under the influence of PHA in 25 CLL patients. Lymphocyte transformation was observed in the cultures for 11 consecutive days (in contrast to the conventional 3-day cultures). This prolonged observation demonstrated that CLL lymphocytes differ functionally from normal lymphocytes in culture not only because they are hyporesponsive to PHA stimulation, but also because the response is delayed. Determinations were obtained prior to and during therapy and were continued during clinical and hematologic remission. These observations allowed us to study the correlation between the degree of lymphocyte transformation and the hematologic and clinical findings. Under adequate therapy, the return to normal transformation of lymphocytes from CLL patients is gradual and the patients reach a hematologic and clinical remission months before the lymphocyte transformation in cultures returns to normal. We also studied the incorporation of tritiated thymidine by the "blastoid" cells in culture and examined the ultrastructure of the transformed normal and CLL blastoid cells.

Materials and Methods

Subjects

The studies were made on a total of 50 individuals: 25 normal controls, consisting of volunteer student nurses and medical students and 25 patient having CLL, including 19 males and 6 females, whose ages ranged from 50 to 80 years.
RESPONSE OF PHA STIMULATED LYMPHOCYTES

Technic of Lymphocyte Culture

Twenty ml. of blood were collected in 2 ml. of dextran and 2 ml. of preservative-free sodium heparin (2000 U.S.P. units per ml.) and allowed to incubate for 2 hours at 37 C. The supernatant plasma was aspirated and leukocyte and supravital differential counts were performed. The sample was then centrifuged at 950 rpm. for 10 minutes. The supernatant was discarded and a calculated amount of 80 per cent minimum essential medium Eagle (MEM), 20 per cent fetal calf serum or autologous plasma and 1 per cent antibiotic solution (200,000 units of penicillin G, 100 mg. of streptomycin and 18,750 units of mycostatin in 10 ml. MEM) were added to the remaining leukocyte pellet. This resulted in a leukocyte concentration of 2,000 cells per cu. mm. in 4 ml. suspension per tissue culture tube. To each tube 0.08 ml. of PHA “M” (General Biochemicals) was added. Unstimulated control cultures were made in the same manner except without PHA. The tubes were incubated in a standing position at 37 C. Differential cell counts of the cultures were made daily for 11 days using both supravital and Wright’s stains. The degree of lymphocyte transformation was expressed as the percentage of blastoid cells among 500 cells counted. Five to ten determinations of different cultures were performed.

Technics for Concentrating Lymphocytes

Two technics for concentrating lymphocytes were used:

1. Blood was collected and sedimented as described for regular lymphocyte cultures. The supernatant was mixed with an equal volume of MEM and 12 ml. of this suspension were placed in siliconized tissue culture bottles. The bottle was laid on its flat side at 37 C. for 45 minutes to allow the polymorphonuclear leukocytes to attach to the glass. The supernatant was then gently poured off and counted. The resulting suspension contained 75 to 85 per cent lymphocytes. The technic for lymphocyte culture was then completed.

2. A total of 400 ml. of blood was collected in a heparinized Fenwal plastic bag (JII-1E) 400 ml. and immediately passed through a Fenwal leukopak filter, designed to remove polymorphonuclear leukocytes, into a transfer pack (TA-4) to which 50 ml. of dextran had been added. The sample was allowed to stand upright for two hours at 37 C. The supernatant plasma was then transferred by means of a plasma extractor to another transfer pack. It was then distributed by gravity from the transfer pack to 40 ml. centrifuge tubes. Differential leukocyte counts were made. The tubes were centrifuged at 950 rpm. for 10 minutes. The plasma was decanted and the pellet of cells was resuspended in the required amount of media to make 2,000 cells per cu. mm. in 4 ml. suspension per tissue culture tube. This technic resulted in a lymphocyte concentration of 98 to 100%.

Technic for Autoradiography

On the third and seventh days of incubation, one tube from each set of cultures was selected and centrifuged for 3 minutes in a table model centrifuge. After 2 ml. of the supernatant was removed with a Pasteur pipette, 0.05 microcuries per ml. cell suspension of 3H-TDR (Thymidine-methyl-H-3, specific activity 6.7 C/mM, New England Nuclear Corporation) was added, mixed and placed in a 37 C. incubator for 45 minutes. The tube was then centrifuged and the supernatant removed. The remaining pellet was washed three times in 2 ml. of cold Hank’s solution. Smears were made from the pellet of cells in the bottom of the tube. The slides were fixed in absolute methanol for 10 minutes. In the dark room, the slides were dipped and coated with NTB-3 (Kodak) liquid emulsion for 15 seconds. After drying, the slides were placed in black, bakelite boxes and stored at 4 C. for 2 weeks. The photographic processing was carried out in the dark room at a temperature of 17.5 to 19.5 C. The slides were developed in D-19 for 2 minutes, fixed in F-5 for 2 minutes and then washed in cold tap water for 5 minutes. They were stained with Harleco Giemsa for 30 minutes and air dried.
RESULTS

Microscopic examination using supravital stain (neutral red-Janus green) was found to be the most accurate method for morphologic differentiation of cells in culture. Wright's stained preparations were also examined, and kept for permanent records.

The blastoid cells were recognized by a well-defined cytoplasmic membrane and a number of features indicating primitivity, including a considerably larger size than the normal lymphocytes. The nucleus had a leptochromatic chromatin pattern and contained one or more well-developed basophilic nucleoli. The cytoplasm was greatly increased in volume. Clear vacuoles, sometimes numerous, were always present in the cytoplasm. The blastoid cells were classified, according to size, as "intermediate" and "large." They were usually observed in clumps, although isolated cells were also seen (Fig. 1).

Cells other than lymphocytes were also seen. These were mainly macrophages, often large, with considerable amounts of phagocytic intracytoplasmic granular debris, copious cytoplasm and ill-defined cytoplasmic membrane. They were readily differentiated from blastoid cells in supravital preparations (Figs. 2 and 3). The small lymphocytes were identified by the usual criteria of size, scanty cytoplasm and deeply staining nuclei (Fig. 4). There were also polymorphonuclear leukocytes, which generally were poorly preserved, clumps of platelets and minimal red cell contamination.
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Fig. 2.—Three day culture of phytohemagglutinin stimulated chronic lymphatic leukemia lymphocytes under supravital-phase microscopy. There are two large blastoid cells and three small lymphocytes. The four macrophages are easily distinguishable from the large blastoid cells by the considerable number of phagocytic intracytoplasmic cells and debris. Magnification × 1,300.

Our studies on the transformation of lymphocytes induced by PHA in 25 normal individuals revealed that, under the conditions of our experiments, the average percentage of transformation of lymphocytes to blastoid cells was 79 after three days in culture. After four and five days, the increase in lymphocyte transformation was minimal with an average of 81 and 80 per cent respectively (Fig. 5). No further increase in transformation was obtained in the succeeding days and the nontransformed lymphocytes degenerated in the cultures. The degree of lymphocyte transformation in normal subjects was highly reproducible.

To determine if autologous human plasma was essential to the system, we made a comparative study utilizing equal proportions of fetal calf serum and homologous normal plasma in place of autologous plasma. The degree of lymphocyte transformation using calf serum and autologous plasma was the same as with homologous plasma.

To ascertain the effect of concentration of lymphocytes in the cultures on the degree of lymphocyte transformation, we conducted a comparative study on normal individuals. One set of cultures was made routinely. In a second set the proportion of lymphocytes was concentrated to 75 to 100 per cent using the technics described. The total leukocyte concentration, 2,000 cells per cu.
Fig. 3.—Three day culture of phytohemagglutinin stimulated normal small leukemia lymphocytes under supravital-phase microscopy. There are three large blastoid cells and three macrophages easily distinguishable because of the considerable amount of phagocytic intracytoplasmic material. Magnification × 1,300.

mm. per 4 ml. culture, remained the same. There was no change in the degree of lymphocyte transformation between cultures.

The results obtained in lymphocyte cultures from 25 untreated CLL patients are presented in Figure 5. The patients were separated into two groups according to the white blood cell count (WBC). The first group consisted of 11 patients with WBC counts of 20,000 to 50,000 per cu. mm. The second group consisted of 15 patients having WBC counts between 50,000 and 500,000 per cu. mm.

Our studies confirmed that the degree of lymphocyte transformation following stimulation with PHA was markedly lower in CLL patients than in normal persons. However, the degree of lymphocyte response to PHA in untreated CLL patients was not always closely related to the WBC count and there were individual variations among patients.

It was also demonstrated that the maximum degree of lymphocyte transformation in both groups of CLL patients was obtained in cultures after 5 to 9 days, with an average of 7 days. Beyond 9 days, there was essentially no increase in the percentage of blastoid cells present. The nontransformed lymphocytes rapidly degenerated. Attempts to prolong the viability of the cultures were made by changing the culture media after 3 to 7 days, but yielded no further increase in the proportion of blastoid cells beyond 9 days.
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Fig. 4.—Seven day culture of phytohemagglutinin stimulated chronic lymphatic lymphocytes observed under supravital-phase microscopy. There are four large blastoid cells and numerous small lymphocytes. Magnification $\times 1,300$.

Fig. 5.—Studies on the transformation of lymphocytes induced by phytohemagglutinin in 25 normal subjects and 25 untreated chronic lymphatic leukemia patients.
This delayed development of maximal proliferation was peculiar to the CLL lymphocyte and was not seen in normal lymphocytes, in which the maximum degree of transformation was reached in three days. These results supported evidence\textsuperscript{12} that the lymphocytes of CLL differ functionally in cultures from normal lymphocytes.

Parallel studies were carried out in patients with CLL by substituting autologous plasma for the same proportion of fetal calf serum or homologous normal plasma in the culture fluid. No difference was found in the degree of lymphocyte transformation between cultures. Conversely, compatible plasma from CLL patients was substituted in normal lymphocyte cultures without influencing the degree of lymphocyte transformation. To determine if higher concentrations of PHA in the cultures could influence the degree of lymphocyte transformation, parallel studies of CLL lymphocyte cultures were observed after the amount of PHA was increased progressively up to eight times the proportion regularly employed. No increase in the lymphocyte transformation was obtained. These experiments suggest that the hyporesponsiveness of CLL lymphocytes is most likely due to a cellular rather than a serum factor.

Unstimulated cultures of normal and CLL lymphocytes were made concomitantly with each one of the experiments described and read daily up to 11 days. The degree of lymphocyte transformation in 74 per cent of the unstimulated cultures was zero, in 15 per cent there were 1 to 3 per cent blastoid cells and in 11 per cent a maximum of 4 to 10 per cent of blastoid cells were found.

Of the 25 CLL patients studied, five have expired. The remaining 20 patients have had long-term follow-up studies of from 3 to 18 months. Prior to therapy, all patients had a complete clinical and hematologic evaluation, and the capability of their lymphocytes to transform into blastoid cells in cultures under the stimulation of PHA was tested. From then on they were checked periodically. All patients were treated initially with leukeran, 6 to 16 mg. daily, and the dose was then gradually decreased and adjusted according to the clinical and hematological improvement. Maintenance leukeran therapy, however, was continued even after they had reached a clinical and hematologic remission.

We were able to correlate the degree of lymphocyte transformation in the cultures with the hematologic and clinical findings. These results are summarized in Figure 6. The patients were divided into three groups according to the current degree of lymphocyte transformation. The first group, 6 patients, has been followed for 7 to 18 months (average 13). The initial WBC counts ranged from 21,400 to 533,000 per cu. mm. (average 134,891), with 74 to 99 per cent lymphocytes (average 90). In 3 days the average initial number of blastoid cells for the patients in this group was 10 per cent, increasing to 17 per cent in 5 days and 30 per cent in 7 days. The current average per cent of blastoid cells for all 6 patients is normal, with 68 per cent in 3 days, with no further increase in transformation in the successive days in culture. All 6 patients are currently in a complete hematologic and clinical remission.
The second group of 6 patients has been followed for periods of 4 to 14 months (average 8). The initial WBC counts were between 14,950 and 160,000 per cu. mm. (average 53,510), with 62 to 95 per cent lymphocytes (average 75). In 3 days the average initial lymphocyte transformation for these patients was 14 per cent, increasing to 20 per cent in 5 days and 24 per cent in 7 days. The current average per cent of blastoid cells for all 6 patients has increased, but has not reached normal levels, with 54 per cent in 3 days, 60 per cent in 5 days and 54 per cent in 7 days. All 6 patients are currently in a complete hematologic and clinical remission.

The third group of 8 patients has been followed for 3 to 16 months (average 8). The initial WBC counts were from 18,800 to 513,000 per cu. mm. (average 148,950), with 63 to 100 per cent lymphocytes (average 81). In 3 days the average initial lymphocyte transformation for the patients in this group was 14 per cent, increasing to 30 per cent in 5 days and 33 per cent in 7 days. The current average lymphocyte transformation for all 8 patients is still abnormal, with a decreased and also a delayed response. The current average number of blastoid cells is 33 per cent in 3 days, 43 per cent in 5 days and 49 per cent in 7 days. None of the patients in this group, at the present time, have reached a complete hematologic or clinical remission.

On the basis of these results, it is apparent that CLL patients receiving leukeran therapy reach a hematologic and clinical remission before the capacity for normal transformation develops. All of the patients were continued on maintenance doses of leukeran even after the hematologic and clinical findings were normal. This was followed by a return of lymphocyte transformation to normal values.

The correlation of lymphocyte transformation in cultures with the clinical and hematologic findings in two CLL patients who had a long term follow-up is presented in Figures 7 and 8.

Figure 7 summarizes the findings in a 64 year old male, who was diagnosed as having CLL by peripheral blood and bone marrow examination on April 5, 1967. The peripheral WBC count was 533,000 per cu. mm. with 99 per cent...
PATIENT No. 10: CHRONIC LYMPHATIC LEUKEMIA

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Fig. 7.—Correlation between the lymphocyte transformation and the clinical and hematologic findings of patient #10.

Lymphocytes. The liver, spleen and lymph nodes were enlarged. The degree of lymphocyte transformation prior to therapy was 4 per cent in 3 days, 8 per cent in 5 days and 17 per cent in 7 days. He was initially treated with leukeran 12 mg. daily. Because of secondary hemolytic anemia, prednisone, 80 mg. daily, was started. The leukeran was decreased to 8 mg. daily and the prednisone progressively decreased and discontinued.

The patient gradually improved, both clinically and hematologically, with an increase in the degree of lymphocyte transformation in the cultures. On May 28, 1968, he had normal clinical and hematologic findings. The number of blastoid cells was increased but not normal, with 50 per cent blastoid cells in 3 days and no delayed transformation of lymphocytes. Four months
later, on September 10, 1968, the degree of lymphocyte transformation reached normal values.

Figure 8 summarizes the findings in a 75 year old female, diagnosed as having CLL by peripheral blood and bone marrow examination on July 5, 1967. The WBC count was 21,400 per cu. mm. with 74 per cent lymphocytes. The spleen and lymph nodes were moderately enlarged. The degree of lymphocyte transformation prior to therapy was 17 per cent in 3 days, 39 per cent in 5 days and 43 per cent in 7 days. Treatment was initiated with leukeran 8 mg. which was progressively decreased to 2 mg. daily.

She made rapid improvement and on September 27, 1967, her clinical and hematologic findings were normal. The degree of lymphocyte transformation increased progressively, but did not reach normal levels until 10 months later.

Our results with comparative autoradiography indicated that normal lymphocytes, after 3 days in culture under the stimulation of PHA, showed a high proportion of cells incorporating tritiated thymidine. After 3 days in culture, CLL lymphocytes showed a much lower proportion of cells incorporating tritiated thymidine. The number of labeled cells in CLL lymphocytes increased after 7 to 9 days in culture. It is noted that the number of labeled cells is proportional to the number of blastoid cells in the culture. Both the intermediate and large blastoid cells were equally capable of incorporating tritiated thymidine (Fig. 9).
Electron microscopic studies of the stimulated lymphocytes are reported in the accompanying article.8

DISCUSSION

The results of our studies are consistent with previous reports indicating that the small lymphocytes of normal human peripheral blood are capable of transformation into blastoid cells when stimulated by PHA in culture.1,3,9 The transformation of normal lymphocytes reaches a maximum three days after culture and there is no significant increase in successive days. The response is not influenced by increasing the proportion of lymphocytes in the cultures, or by substituting autologous plasma for the same proportion of homologous plasma or fetal calf serum.

Our studies also support previous reports indicating that the transformation of CLL lymphocytes in culture is significantly decreased in comparison to the transformation of lymphocytes from normal subjects.2 The hyporesponsiveness of CLL lymphocytes is apparently related to cellular rather than to serum factors. This is indicated by the inability of fetal calf serum or homologous normal plasma to enhance the CLL cell response, and the inability of CLL plasma to inhibit the degree of transformation of normal lymphocytes in culture.

Lymphocytes of CLL also differ from normal lymphocytes because their response to PHA stimulation is delayed in culture. The maximum response of CLL lymphocytes was obtained in 5 to 9 days with an average of 7 days. The maximum response of normal lymphocytes was obtained in 3 days, and from then on no significant increase in blastoid cells was noted. This finding of a delayed response of CLL lymphocytes was further supported by tritiated thymidine autoradiography studies, which showed higher numbers of tagged cells in 7-day cultures. The delayed response of CLL lymphocytes had been suggested by Oppenheim11 and demonstrated by Havemann and Rubin.12

Our results indicate that while CLL and normal lymphocytes are morphologically similar, lymphocyte transformation in response to PHA stimulation provides an effective in vitro method for studying their functional differentiation.

Even when the WBC counts were above 100,000 per cu. mm. and the percent of lymphocytes was 88 to 99, the 25 untreated CLL patients still had some lymphocytes which reacted normally to stimulation with PHA. This could be interpreted as supporting previous reports suggesting that two distinct populations of lymphocytes, normal and neoplastic, coexist in CLL.4,6,13

In the long-term follow-up study of CLL patients before and during therapy, the clinical and hematologic remission preceeded the return of lymphocyte transformation to normal in vitro, sometimes by several months. It may be inferred that CLL patients have a mixed population of lymphocytes. The neoplastic lymphocytes may be more sensitive to leukeran therapy, be progressively inhibited, and then destroyed while the population of normal lymphocytes increases and finally predominates.

Our observations suggest that leukeran produced a return of in vitro
lymphocyte response to PHA stimulation to normal as well as a total reduction of WBC count. With total body irradiation a complete return of lymphocyte transformation to normal has been reported in three patients. Since clinical and hematologic findings return to normal before the lymphocyte transformation becomes normal, our observations might also suggest that maintenance levels of leukeran therapy should be prolonged beyond the time of clinical and hematologic remission. This in vitro system could be used as a test for the comparative study of the efficacy of therapeutic agents employed in the treatment of CLL.

Although the mechanism of lymphocyte transformation in vitro under the influence of PHA is not fully understood, it has been shown to be impaired in lymphocytes of patients with diseases where immunity is also impaired. Some authors have suggested that PHA is an antigenic stimulus, while others favor the view that PHA acts as a nonspecific inducer of growth. Therefore, the altered function of CLL lymphocytes in vitro could reflect either an immune defect or an impaired response on the basis of metabolic derangement.

Although not yet fully understood, the behavior of lymphocytes in short-term tissue culture is one of the most promising in vitro systems for the study of lymphocyte function. It may also offer new parameters in evaluating response to therapy.

**SUMMARY**

Comparative studies of in vitro responses to PHA stimulation between normal lymphocytes and lymphocytes of CLL have shown significant functional differences.

Lymphocytes from 25 normal subjects showed maximum response at three days, while the leukemic cells did not reach a maximum degree of transformation until seven days. Moreover, the maximum transformation of the leukemic lymphocytes was significantly less than that of the normal lymphocytes.

After therapy, the lymphocytes of the leukemic patients showed a gradual return to the levels and rates of lymphocyte transformation of the normal controls. This generally followed hematologic and clinical remission by several months.

This in vitro system demonstrates functional differences between normal lymphocytes and the lymphocytes of CLL. It could also be used as one method for judging the therapeutic efficacy of agents used in the treatment of CLL.

**SUMMARIO IN INTERLINGUA**

Studios comparative del responsas in vitro a stimulation per phytohemagglutinina de lymphocytos normal e de lymphocytos ab chronic leucemia lymphocytic ha revelate significative differentias functional.

Lymphocytos ab 25 subjectos normal manifestava le responsa maxime post tres dies, durante que le cellulas leucemic non atingeva le grado maxime de lor transformation usque al fin de un intervallo de septe dies. In plus, le transformation maxime de lymphocytos leucemic esseva significativemente inferior a illo de lymphocytos normal.
Post le therapia, le lymphocytos del patientes leucemic monstrava un retorno gradual al nivellos e proratas del transformation lymphocytic del controlos normal. Isto sequeva in general le remission hematologic e clinic per plure menses.

Iste systema in vitro demonstra differentias functional inter lymphocytos normal e lymphocytos de chronic leucemia lymphocytic. Illo etiam poterea esser usate como un metodo pro judicar le efficacia therapeutic de agentes usate in le tractamento de chronic leucemia lymphocytic.

REFERENCES

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