Clin. exp. Immunol. (1974) **16,** 553–563.

CHRONIC LYMPHOCYTIC LEUKAEMIA

STUDIES ON MITOGEN-STIMULATED LYMPHOCYTE INTERFERON AS A NEW TECHNIQUE FOR ASSESSING T LYMPHOCYTE EFFECTOR FUNCTION

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(*Received* 2 *August* 1973)

SUMMARY

The present study employs a new technique for the study of human T-cell effector function in patients with chronic lymphocytic leukaemia: mitogen-stimulated interferon. Cultures of macrophages, T cell-enriched lymphocytes, or macrophages and lymphocytes combined were prepared from the blood of fourteen normal donors and five patients with chronic lymphocytic leukaemia. The effects of the mitogens, phytohaemagglutinin and pokeweed, on interferon production and lymphocyte transformation were studied and the following observations made: (a) T-cell effector and proliferative functions were depressed as evidenced by the absence of interferon and proliferative response to PHA and PWM at 3 days *in vitro*; (b) Three out of five patients showed no interferon or proliferative response at 6 days, thus indicating a B lymphocyte abnormality as well; (c) macrophages from both normal and leukaemic subjects augmented mitogen-stimulated lymphocyte interferon production and lymphocyte transformation. However, the addition of normal allogeneic macrophages to cultures of lymphocytes prepared from the patients did not restore the proliferative and interferon responses to normal levels.

INTRODUCTION

Interferon is a mediator of cellular immunity. It is produced *in vitro* after leucocytes are stimulated with the nonspecific mitogens, phytohemagglutinin (PHA) (Wheelock, 1965) and pokeweed mitogen (PWM) (Friedman & Cooper, 1967), after immune specific stimulation of leucocytes by various antigens (Green, Cooperband & Kibrick, 1969), and as a by-product of the mixed lymphocyte reaction (Gifford, Tibor & Peavy, 1971). In addition, interferon (in contrast to the other mediators of cellular immunity) has also been demonstrated to occur *in vivo*, after stimulation with PHA (Epstein & Merigan, unpublished observations) and a specific antigen (Stinebring & Absher, 1970).

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Our own studies have identified the lymphocyte as the cell type in the human which is responsible for the *in vitro* production of interferon secondary to stimulation with mitogen (Epstein, Cline & Merigan, 1970, 1971a) and bacterial (Epstein, Cline & Merigan, 1971b, 1972) and viral antigens (Epstein, Stevens & Merigan, 1972; Merigan *et al.*, 1973). Furthermore, we demonstrated that the addition of macrophages to cultures of lymphocytes markedly augmented the amount of interferon produced by the lymphocytes in response to these agents.

Recently, in an attempt to learn more about interferon production as a manifestation of the effector function of human lymphocytes we employed a new and unique instrument, a fluorescence-activated cell sorter, to isolate highly purified preparations of human T and B lymphocytes (Epstein, Kreth & Herzenberg, 1974). Using such purified preparations we demonstrated that in the human both T and B lymphocytes can proliferate and produce interferon *in vitro* in response to PHA and PWM in the presence of macrophages. However, the proliferation and production of interferon by mitogen-stimulated T cells was observed at 3, 5, and 7 days in culture, whereas that observed in cultures of B cells was delayed and only observed at 5 and 7 days. Thus the study of mitogen-stimulated interferon production at 3 days can be used as a technique for the evaluation of the competence of T-cell effector function, as well as for the better known proliferative response. Comparable studies of interferon production and proliferation at 5–7 days reflects the activity of both cell types.

Our own study (Epstein, Kreth & Herzenberg, 1974) and that by others (Eisen, Wedner & Parker, 1972), indicated that the passage of mixed human T- and B-lymphocyte populations through a nylon fibre column resulted in an effluent highly purified for T cells. The peripheral blood of most patients with chronic lymphocytic leukaemia represents a rich source of lymphocytes which bear surface-bound immunoglobulins and, by analogy with observations in the mouse, designate them as belonging to the B or bone marrow-derived lymphocyte population (Papamichail, Brown & Holborow, 1971; Froland, Natvig & Berdal, 1971; Johannson & Klein, 1970; Grey, Rabellino & Pirofsky, 1971; Unanue *et al.*, 1971). Recently, it was shown that 75% of lymphocytes from patients with chronic lymphocytic leukaemia adhere to nylon fibre columns whereas only 41% of normal lymphocytes adhere (Boldt, Skinner & Kornfeld, 1972).

Thus, in the present study we used nylon fibre columns to prepare T cell-enriched populations of lymphocytes from normal donors and patients with chronic lymphocytic leukaemia in an attempt to answer the following questions. (1) Is T-lymphocyte effector function, as manifested by the production of interferon at 3 days in response to PHA and PWM, normal in patients with chronic lymphocytic leukaemia? (2) If abnormalities in mitogen-stimulated T lymphocyte interferon production are detectable, are they related to the well documented depression of mitogen-stimulated lymphocyte transformation in such patients (Nowell, 1960; Shrek & Rabinowitz, 1963; Quaglino & Cowling, 1964; Astaldi *et al.*, 1965; Oppenheim, Whang & Frei, 1965; Smith, Cowling & Barker, 1972; Catovsky, Tripp & Hoffbrand, 1972; Konig *et al.*, 1972; Froland & Stavem, 1972)?

MATERIALS AND METHODS

Subjects

Five patients with chronic lymphocytic leukaemia were studied, one of them on two occasions. The duration of illness was between 1·5 and 4·5 years. The diagnosis of chronic

lymphocytic leukaemia was established by the characteristic infiltration of bone marrow with large numbers of lymphocytes and by an elevated peripheral white blood count with a marked preponderence of lymphocytes. None of the patients was receiving chemotherapy at the time of this study, and all had been off therapy for 6 months or more prior to the initiation of the study. Fourteen healthy adults with normal w.b.c. and differential counts and no recent history of viral illness served as controls.

Preparation of PHA

PHA-P (Difco Laboratories, Detroit, Michigan) was prepared as previously described (Epstein *et al.*, 1971a). Three doses were employed, giving a final concentration in culture of 17, 33, or 50 μg/ml.

Preparation of PWM

The contents of each vial of PWM (Grand Island Biological Company, (GIBCO), Berkeley, California) were dissolved in 5 ml of sterile phosphate-buffered saline, pH 7·4 (GIBCO). A 1/10 dilution of this solution was prepared and frozen in aliquots for use as desired. Three doses were employed, giving a final concentration in culture of 24, 45, or 84 μg/ml.

Preparation of cultures

Macrophage cultures were obtained by the growth and differentiation for 7 days of glass-adherent monocytes in Leighton tubes containing AB serum-enriched McCoy's medium, as previously described (Epstein *et al.*, 1971a). The method was modified as follows: leucocyte preparations containing $1{\cdot}1 \times 10^6$ monocytes were added to small Leighton tubes (16×85 mm) in 0·75 ml of medium. After a 2-hr incubation at 37°C, the nonadherent cells were decanted and the cultures treated as previously described. The resulting macrophage cultures were 96–100% pure.

Before use, each culture was examined microscopically and evaluated for cell number and extent of differentiation. Representative cultures prepared from normals were counted by a method described previously (Epstein *et al.*, 1971a). In such cultures, the final concentration of macrophages was $2\text{–}5 \times 10^4$/ml.

Unless otherwise stated, blood for the isolation of lymphocytes was always obtained from the same donor who 1 wk previously had donated blood for the preparation of macrophage cultures. Lymphocytes were obtained by their passage through a sterile nylon fibre column (Epstein *et al.*, 1971a), and the resulting preparations were 99·5–100% pure. Erythrocytes that accompanied the lymphocytes in the column effluent were lysed by exposure to NH_4Cl (Epstein *et al.*, 1972). Purity of the lymphocyte suspensions was determined for each experiment by morphological criteria. In numerous instances the lack of phagocytosis of heat-killed *C. albicans* was used as an additional criterion for identification of lymphocytes (Epstein *et al.*, 1971a).

Our previous study (Epstein *et al.*, 1973) had shown that the nylon fibre column cell effluent prepared from normal donors contained only 4–9% (mean 6·5%) B cells after being stained with fluorescein-labelled anti-Cohn Fraction II. The B-cell nature of such cells obtained from normal donors was confirmed by the presence of surface bound immunoglobulin and by the development of such cells into mature plasmacytes after prolonged culture with PWM (Kreth & Herzenberg, 1974). For the present study the cell

effluents from two patients with chronic lymphocytic leukaemia were monitored for the presence of surface immunoglobulin, using the same fluoresceinated antisera. The results indicated that such effluents contained 40% and 22% cells with several areas of bright fluorescence, and hence were considered to be B cells. Presumably, the remainder of the cells, the non-immunoglobulin-bearing ones, or 60% and 78% respectively, were T cells.

Thus, from each donor it was possible to prepare T lymphocyte-enriched cultures, macrophages, or the two cell types together with PHA, PWM, or no stimulant added at the initiation of the cultures. The final volume of each culture was 1 ml, and the concentration of lymphocytes 9×10^5/ml. The concentration of macrophages was $2–5 \times 10^4$ ml in cultures prepared from normals and two patients. Fewer macrophages were present in cultures from the other donors.

In two experiments allogeneic cultures were prepared with normal macrophages plus patients' lymphocytes. In comparable control experiments macrophages from two normal donors were combined with lymphocytes from the same donors in allogeneic and autochthonous mixes.

Harvesting techniques

The cultures were maintained at 37°C in a CO_2 incubator for 3 and 6 days. [^{3}H]Thymidine (New England Nuclear Company, Boston, Massachusetts; specific activity 2·1 Ci/mmole) was added 1 hr before termination of each culture. The degree of incorporation of [^{3}H] thymidine into DNA was used as a measure of the extent of lymphocyte transformation (Epstein *et al.*, 1972).

Interferon assay

A plaque reduction assay employing human neonatal foreskin fibroblasts and bovine vesicular stomatitis virus was employed (Epstein *et al.*, 1971a; Merigan, Gregory & Petralli, 1966). Interferon titre of supernatant culture fluid was defined as that dilution of sample which, in a 4–ml volume, resulted in 50% reduction of viral plaques. In most cases, duplicate or triplicate samples were run for each type of culture. Control supernatants with medium plus PHA or PWM were also tested. At least two, and most often three, dilutions of each sample were run along with the controls for the interferon assay; i.e., fibroblast monolayers with virus and no sample to be tested, and fibroblast monolayers with virus and dilutions of a known interferon standard. For statistical purposes interferon titres of <10 were assigned a value of 5.

RESULTS

General observations on the macrophage cultures

The macrophages cultures prepared for this study depended on the growth and differentiation of glass-adherent monocytes. Monocytes comprised about 5–15% of the cells in the leucocyte-rich plasma of normal donors. Therefore, in the preparation of normal macrophage cultures the initial cell inoculum consisted of $1·1 \times 10^6$ monocytes plus $6·2–20·9 \times 10^6$ other leucocytes. The macrophages that resulted after 7 days were well differentiated and appeared as large cells with eccentric nucleic and foamy cytoplasm, and many were elongated. With three of the patients, however, monocytes comprised only 0·5–1% of the cells found in the leucocyte-rich plasma. Therefore, those initial cell inocula contained

$1{\cdot}1 \times 10^6$ monocytes and up to 219×10^6 other leucocytes (mainly leukaemic lymphocytes). The macrophages that resulted were small and poorly differentiated and were few in number. The paucity of macrophages resulted from the cell crowding and diminished access to the Leighton tube coverslip, which were associated with the increased size of the initial cell inoculum. This was confirmed by an experiment in which the initial inoculum of monocytes was derived half from the blood of a control donor (from whom excellent macrophage cultures had been prepared) and half from the blood of a patient which contained large numbers of lymphocytes. Under these conditions, the resulting macrophages were low in number and poorly differentiated. However, it is possible that immunological reactions in the mixed cell cultures might have contributed to the failure of attachment of the normal donor's monocytes.

Effect of PHA on lymphocyte transformation and interferon production in combined autochthonous lymphocyte-macrophage cultures

Fig. 1a depicts the effect of PHA on lymphocyte transformation in the combined cultures at 3 and 6 days. Fourteen normal donors were studied but in some instances not all doses of PHA were employed, and only 6 donors were studied at 6 days. Each point represents the mean of duplicate or triplicate cultures (from which has been subtracted the background counts from cultures prepared without mitogen). The range of values for normal donors was very wide. A somewhat more limited range of values for the normals was observed at 6 days. However, the results obtained from the studies with the patients' blood were in marked contrast to the normals. At 3 days all values were lower than the range observed for normals, demonstrating a marked decrease in T-lymphocyte transformation. At 6 days, all patients demonstrated slightly higher values than observed at 3 days. However, only

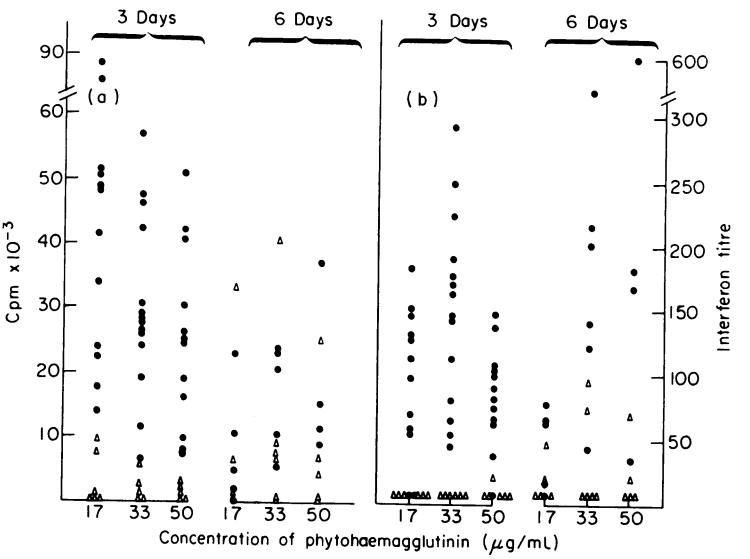

FIG. 1. Effect of PHA on (a) lymphocyte transformation, measured by [^{3}H]thymidine incorporation and (b) interferon production in combined autochthonous lymphocyte–macrophage cultures at 3 and 6 days. (●) Normal donors. (△) Patients with chronic lymphocytic leukaemia. Cpm = counts per minute ((M + L + PHA)-(M + L)).

one patient clearly demonstrated a significant delayed response, with values observed in the mid-range of that observed for normals at 3 days.

Fig. 1b depicts the interferon titres observed in the supernatants of the combined cultures. Again, as was the case with degree of lymphocyte transformation, a wide range of interferon titres was observed for normal donors. But clearly, at 3 days no detectable T-lymphocyte interferon was noted in the cultures prepared from patients. By 6 days, however, one patient had a response comparable to that observed for normals at day 3.

Effect of PWM on lymphocyte transformation and interferon production in combined autochthonous lymphocyte–macrophage cultures

The extent of PWM-stimulated lymphocyte transformation in combined cultures is depicted in Fig. 2a. The range of values noted for the normal donors at day 3 was wide, but more circumscribed than the response observed with PHA. All of the values observed for the patients fell well below the normal range except for those of two patients who demonstrated a low normal response but only at one of the three doses of PWM employed. At 6 days the same situation obtained; there was a depression in lymphocyte transformation in all cultures prepared from the patients, with the exception of a few of those prepared from two patients.

All the normal donors studied produced T-lymphocyte interferon in response to PWM (Fig. 2b). Most of the donors responded with high titre by day 3, although two of the twelve donors did not respond significantly until 6 days. No interferon was noted in any of the PWM-stimulated cultures derived from the patients at 3 and 6 days, with few exceptions.

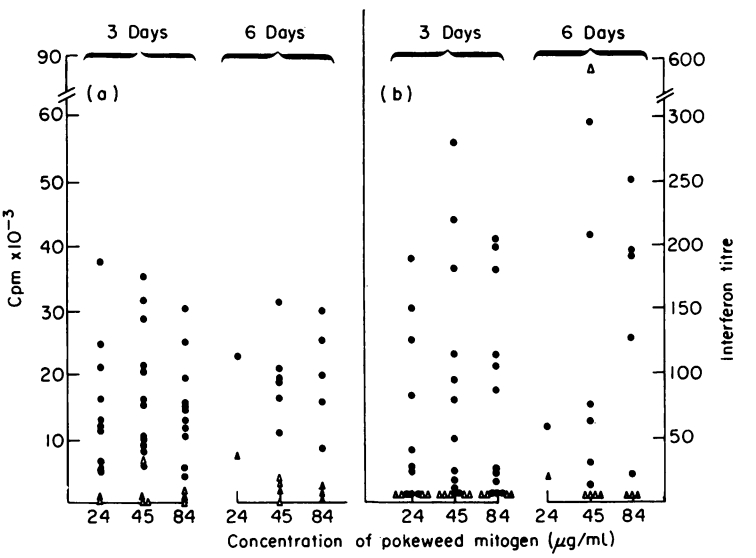

FIG. 2. Effect of PWM on (a) lymphocyte transformation, measured by [^{3}H]thymidine incorporation and (b) interferon production in combined autochthonous lymphocyte–macrophage cultures at 3 and 6 days. (●) Normal donors. (∧) Patients with chronic lymphocytic leukaemia. Cpm = counts per minute ((M+L+PWM)-(M+L)).

No interferon was ever detected in lymphocyte or combined lymphocyte–macrophage cultures prepared without PHA or PWM from either patients or control subjects. Also, background [^{3}H]thymidine incorporation was equivalent in unstimulated cultures prepared from patients and controls. In both, the 6-day values were usually higher than the 3-day values for combined lymphocyte–macrophage cultures.

Physical and chemical characterization of the interferon produced in combined cultures

The techniques employed to characterize interferon have been described previously (Epstein *et al.*, 1971a). The interferon observed in the present study was species specific, was not sedimented by centrifugation at 100,000 *g* for 1 hr, was susceptible to the action of trypsin, and was partially inactivated by heating at 56°C for 1 hr. Exposure of the culture supernatants to pH 2 for 18 hr resulted in considerable loss of activity. The fluids did not directly inactivate vesicular stomatitis virus. Furthermore, exposure of the confluent monolayers of neonatal foreskin fibroblasts to the supernatant fluids for only 2 hr instead of the customary 18–24 hr was not sufficient time for the interferon to exert its protective effect. The sensitivity to low pH and heat differentiates the interferon produced in response to mitogens from that produced by viruses (Wheelock, 1965). Viral-stimulated interferon is not inactivated by heating at 56° for 1 hr or pH 2 for 18 hr.

Effect of autochthonous macrophages on PHA- and PWM-stimulated lymphocyte transformation and interferon production

To determine the ability of autochthonous macrophages to augment PHA- and PWM-stimulated lymphocyte transformation and interferon production, the results of cultures containing macrophages and lymphocytes with stimulant were compared with those containing only lymphocytes plus stimulant. A two-fold increase or greater was considered to represent significant augmentation of lymphocyte transformation or interferon production. At 3 days the presence of macrophages in cultures derived from normal donors always resulted in the augmentation of PHA-stimulated lymphocyte transformation (3·4-fold mean increase) and interferon production (8·1-fold mean increase). By the 6th day lymphocyte transformation in cultures from normals was on the wane, and the presence of macrophages had little effect on transformation (1·6-fold mean increase) but did have a major effect on interferon production (32-fold mean increase).

Whereas the effect of macrophages on PWM-stimulated lymphocyte transformation was most noticeable at 3 days (26-fold mean increase), the augmentation of PWM-stimulated interferon was more pronounced at 6 days (19-fold mean increase). Examination of the data obtained from the cultures prepared from three of the patients revealed no significant effect of macrophages on either lymphocyte transformation or interferon production at either 3 or 6 days. This can probably be explained by the scanty number and poor differentiation of the macrophages prepared from these particular donors. When satisfactory macrophage preparations were obtained from patients with chronic lymphocytic leukaemia (as was the case with the other two patients), the macrophages were capable of augmenting transformation and interferon production. Their effect on transformation was delayed, however; with PHA the augmentation was greater at 6 days (47-fold mean increase) than at 3 (23-fold mean increase). Similarly their effect on PHA-stimulated interferon production was greater at 6 days (17 fold mean increase) than at 3, where there was no effect.

It is important to note, however, that cultures that contained only patients' lymphocytes and mitogens uniformly had less transformation than that observed with normals, and interferon was never detected.

Effect of allogeneic macrophages on PHA- and PWM-stimulated lymphocyte transformation and interferon production

To determine if the depressed or delayed lymphocyte transformation and interferon production observed in the cultures from patients with chronic lymphocytic leukaemia could be corrected by the addition of normal macrophages, cultures were prepared with normal allogeneic macrophages and patients' lymphocytes. We had established in normals that the degree of lymphocyte transformation and interferon production was a function of the lymphocytes employed and not of the macrophages, and that interferon would not be produced as a by-product of an allogeneic lymphocyte-macrophage culture without PHA stimulation. In two experiments in which normal macrophages were added to two patients' lymphocytes, no improvement in PHA- or PWM-stimulated lymphocyte transformation or interferon production was noted. The data from one such experiment is shown in Table 1. Thus, the substitution of abundant numbers of well differentiated normal macrophages could not correct the depressed T-cell proliferative and interferon responses observed in the cultures prepared from the patients.

TABLE 1. The effect of normal allogeneic macrophages on PHA- and PWM-stimulated lymphocyte transformation and interferon production by chronic lymphocytic leukaemic lymphocytes

Culture type*	Cpm†	Interferon titre
3-day cultures		
M_p+L_p	1589	<10
M_p+L_p+PHA	1,326	<10
M_p+L_p+PWM	568	<10
M_n+L_n	266	<10
M_n+L_n+PHA	47,589	246
M_n+L_n+PWM	9689	30
M_n+L_p	171	<10
M_n+L_p+PHA	759	<10
M_n+L_p+PWM	113	<10

* The subscripts identify the source of the macrophages and lymphocytes, p being from a patient and n from a normal.

† The results expressed are the mean values of duplicate cultures.

DISCUSSION

The present studies demonstrate that there is a diminished ability of T lymphocytes from patients with chronic lymphocytic leukaemia to produce interferon in response to PHA and PWM at 3 days. To a degree, the depressed response in interferon production paralleled that of T-lymphocyte transformation in that no interferon was produced in

response to these agents in the absence of significant transformation. The depressed interferon response was most pronounced at 3 days and was demonstrated for both PHA and PWM with all five patients studied. At 6 days, one of the patients demonstrated a significant response. Another patient demonstrated a significant but only partial response at 6 days in that only some of the doses of PHA and PWM resulted in significant levels of lymphocyte transformation and interferon production. It is of interest to note that both of these pateints had the shortest duration of illness and the lowest peripheral blood leucocyte count.

The present study demonstrates therefore that T-cell proliferative response and T-cell effector function is deficient in all of these patients. Furthermore, the absence of a 6-day response by three patients also suggests that their B-cell proliferative ability and effector functions are abnormal. The response of the other two patients at 6 days could be attributed either to a delayed T-cell response or a normal B-cell response.

There are several possible explanations for the depressed, mitogen-stimulated T-lymphocyte interferon response observed in the present study. First, there might not be enough T cells in the column effluents to produce detectable levels of interferon. This is unlikely, as in the two effluents from the patients which were monitored by fluorescence microscopy, 60 and 78% of the cells were non-immunoglobulin-bearing, or T lymphocytes. Furthermore, in previous studies we demonstrated that as few as $2{\cdot}5 \times 10^4$ normal T lymphocytes can produce detectable levels of interferon when stimulated with PHA or PWM, and the cultures in the present study contained more than twenty times that number.

Second, the presence of leukaemic B cells may in some way inhibit the normal function of T lymphocytes. At present this cannot be confirmed until techniques for the separation of leukaemic from normal B cells are developed. We do know however that the presence of normal B cells does not inhibit the T-cell response (Epstein *et al.*, 1974). Furthermore, until techniques for separating normal B from leukaemic B cells become available we will not be able to resolve whether the depressed interferon response seen in some of the patients at 6 days is a manifestation of the diluting out of normal B cells by leukaemic cells and/or the inability of leukaemic B cells to produce interferon. Robbins & Levis (1972) have suggested that there is an inherent inability of leukaemic lymphocytes to respond to PHA.

Another possible explanation for the depressed interferon response might be an inability of macrophages from patients with chronic lymphocytic leukaemia to augment the production of interferon. This is unlikely as was shown in the two instances in which abundant and well-differentiated macrophage preparations were obtained from the blood of patients. Such macrophage preparations were capable of augmenting to some degree both interferon production and lymphocyte transformation. Moreover, even normal allogeneic macrophages could not restore the patients' T-cell interferon production and transformation to normal levels.

Finally, there might actually be a defect in T-lymphocyte proliferative and effector functions in patients with chronic lymphocytic leukaemia. At present we subscribe to this explanation because of the data herein. It is of interest that Wybran, Chantler & Fudenberg (1973) recently reported normal proliferative response to PHA by T cells isolated by a rosette-forming technique from the blood of patients with chronic lymphocytic leukaemia. However, the responses they noted peaked at 5 days, instead of the customary 3 days which is the case for normal T cells (MacKinney & Stohlman, 1961) found by most investigators.

Defects in the ability of leucocytes from patients with chronic lymphocytic leukaemia to produce interferon in response to agents other than mitogens have been described previously. Lee, Ozere & van Rooyen (1966) found that the *in vitro* interferon response of Sendai virus-treated leucocytes from seven patients with chronic lymphocytic leukaemia was markedly depressed when compared with cultures of Sendai virus-treated leucocytes of normal subjects. In another study of 12 patients with the disease the same authors found a low yield of Sendai virus-induced leucocyte interferon in those patients with a duration of disease of 14–15 years, and normal levels of interferon in those patients with a duration of disease of only 1 month to 3 years (Lee, van Rooyen, & Ozere, 1969). Also, Hadhazy *et al.* (1967) found that leucocytes from three of four patients with chronic lymphocytic leukaemia had decreased interferon response to Sendai virus *in vitro*, and two of three had a decreased response to Newcastle disease virus. These studies suggest, as does the present study, that the longer the duration of illness and the higher the leukaemic white cell count, the more severe the depression in *in vitro* interferon production, whether it be in response to virus or to the mitogens, PHA and PWM.

ACKNOWLEDGMENTS

This work was supported by the Damon Runyon Memorial Fund, Grant DRG-1205; by U.S. Public Health Service Grants CA 11067 and 12822; and by the American Cancer Society, California Division, Special Grant number 605.

Our sincerest thanks to Mrs Norma Kent and Mrs Lisken Byers for excellent technical assistance. Also, we thank Mrs Ruth Waters in Dr Leonard Herzenberg's laboratory at Stanford for performing the immunofluorescence staining and microscopy.

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