Interacting Cell Populations in Cultures of Leukocytes From Normal or Leukemic Peripheral Blood

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Kinetic studies in cultures containing $2 \times 10^5$ peripheral leukocytes from patients with acute myeloblastic leukemia revealed extensive, radiation-sensitive increases in thymidine incorporation without parallel increases in cell number. Modest and variable stimulation of $\textsuperscript{3}H\text{dR}$ incorporation was seen with the addition of either leukocyte-conditioned medium prepared with phytohemagglutinin (PHA) or PHA alone. However, using the method of limiting dilution, stimulation was always observed and ranged from 3- to 20-fold in individual patients. By mixing small numbers of intact cells with larger numbers of irradiated autologous cells, quantitative evidence was obtained for a cellular interaction between irradiated, PHA-stimulated populations and a subpopulation capable of $\textsuperscript{3}H\text{dR}$ incorporation. Similar evidence for cell-cell interaction was obtained for normal leukocytes.

Previous studies of progenitor cells in cultures from marrow or peripheral blood of patients with leukemia have led to the suggestion that leukemic proliferation might not be autonomous, but instead subject to regulation. Support for the hypothesis has been obtained from kinetic studies on cultures of cells from the peripheral blood of patients with leukemia where the peripheral blast count was high. The data were interpreted in terms of a model. It was postulated that leukemic peripheral blood contained at least three populations: a large number of relatively inert blast cells, a small number of cells capable of proliferation, and a population that modulated proliferation by releasing stimulatory substances, particularly in the presence of the lectin phytohemagglutinin (PHA).

The model was based on studies of cells from certain patients; for these cell populations, kinetic data provided clear evidence for stimulation of proliferation by substances released from either normal or leukemic leukocytes, including a subpopulation coexisting with proliferative cells in leukemic blood. Similar kinetic studies on cell populations from other patients yielded little or no evidence of stimulation by leukocyte-derived factors.

The present paper contains an approach to the demonstration of cellular interactions in cell populations from the latter type of patient, based on culturing leukocytes over a wide range of cell concentrations. Evidence for cellular interactions has been obtained not only for cultures of leukemic leukocytes, but...
also for normal peripheral blood cells, as might be anticipated if physiological regulatory mechanisms persist in leukemia.

MATERIALS AND METHODS

Cells, from either patients with leukemia or normal volunteers, were obtained by venipuncture using heparin as an anticoagulant. The leukocytes were separated from erythrocytes and either tested directly or frozen at −70°C in 20%, fetal calf serum with 5%, DMSO, as described previously. Results obtained on fresh or preserved specimens were qualitatively similar, and the freezing procedure regularly permitted survival of approximately 80%, of activity. In the present series, fresh and preserved leukocytes were not compared routinely. However, in three instances, such comparisons showed qualitatively similar kinetics and between 60%, and 80%, survival of 3HTdR incorporation at the peak. Leukocyte-conditioned media (LCM) were prepared by incubating 10⁷ normal or leukemic leukocytes in 20%, fetal calf serum with medium without nucleosides or nucleotides (growth medium, GM) and 1%, v/v phytohemagglutinin (PHA) (Burroughs Wellcome). Cultures consisted of 3-ml cell suspensions, at appropriate cell concentrations, in growth medium with additions dictated by the specific requirements of individual experiments. For PHA, preliminary dose response curves were obtained; these indicated that 0.5% (v/v) was optimal, with inhibition often occurring at greater concentrations. Therefore, 0.5%, PHA was used throughout. Activity of leukocyte-conditioned media varied from preparation to preparation. The optimum concentration for each preparation was determined before its use, and each preparation was used at that concentration thereafter. The preparation-to-preparation optimum concentration varied from 5% to 20%. These cultures were incubated at 37°C in a moist atmosphere with 5% CO₂ for varying times, depending upon the kinetic parameters of the cells and the design of individual experiments. After incubation, the cultures were pulse-labeled with high specific activity tritiated thymidine (3HTdR) for either 2 or 4 hr. Incorporation was stopped by the addition of cold thymidine, and material insoluble in trichloroacetic acid (TCA) was collected on Millipore filters. Incorporation of 3HTdR was linear with time for up to 4 hr. Two-hour pulses were used in kinetic experiments for cultures at a density of 2 × 10⁵ cells/ml, since under these conditions adequate incorporation above background was observed. For experiments with cultures at varying, but low cell densities, 4-hr pulses gave improved discrimination between incorporation and background. These were counted in a liquid scintillation counter, and the results were expressed as counts/min/culture. When required, cells were irradiated using a 137Cs irradiator and pulse labeled with 3HTdR 7-12 days later in the usual way.

RESULTS

Leukocytes from patients with leukemia or normal donors were preserved by freezing and studied using three experimental designs: (1) the kinetics of thymidine incorporation were obtained in order to determine an appropriate time for (2) measurement of the effect of varying cell numbers; and (3) the relationship between cell number and thymidine incorporation was used in mixing experiments designed for the demonstration of cellular interactions.

Kinetics

Figure 1 depicts the kinetics of thymidine incorporation and nucleated cell number for leukocytes obtained from a patient, R, with acute myelogenous leukemia (AML) and cultured at 2 × 10⁵ cells/ml. Thymidine incorporation increased approximately 20-fold with a maximum after 5-8 days in culture. The increase was not associated with a change in nucleated cell number and was abolished by irradiation with 1000 rads. Under the conditions of Fig. 1, no stimulation was observed in cultures containing leukocyte-conditioned medium (LCM), although the same LCM preparation was active in stimulating
Fig. 1. Kinetics of $^3$HTdR incorporation (top panel) and of nucleated cell number (bottom panel) in cultures containing $2 \times 10^5$ nucleated cells/ml from a patient, R, with acute myeloblastic leukemia. e, cultures in growth medium (20% fetal calf serum and no medium); o, cultures in growth medium supplemented with leukocyte-conditioned medium prepared in the presence of PHA. Data are pooled from two experiments. Error bars represent standard errors.

thymidine incorporation by cells from other patients. In seven additional patients with AML and one with chronic myelogenous leukemia (CML), 2- to 30-fold increases in thymidine incorporation were observed in cultures with growth medium alone and modest, variable stimulation (two- to fourfold) with the addition of leukocyte-conditioned media or phytohemagglutinin.

Cell Number Variation

Occurrence of extensive, radiation-sensitive increases in thymidine incorporation without parallel changes in cell number was consistent with the concept that proliferation was occurring in a minority subpopulation. Confirmation of this view was sought using the method of limiting dilution. Fifteen to twenty cultures containing the same small number of cells were scored as either positive or negative. In preliminary experiments, no relationship was observed between $^3$HTdR incorporation and cell number when incorporation was less than

Fig. 2. Results of limiting dilution experiments on peripheral leukocytes from patient R. Each point is derived from 15 to 20 replicate cultures. Data are pooled from five experiments. o, cultures in growth medium alone; e, cultures supplemented with leukocyte-conditioned medium.
10^3 counts/min, and, accordingly, cultures with ^3HTdR incorporation in excess of this value were considered positive. Figure 2 contains the pooled results from five experiments with cells from patient R. It was evident that the cell number required for 50\%, positivity differed for cultures in growth medium compared to cultures with added LCM. These data provided approximate estimates of the frequency of proliferating units in the population in the presence or absence of stimulator. It should be noted that the limiting dilution data showed a transition from 0\% to 100\% positivity over a narrower range of cell number than would be expected from "single event" Poisson statistics. This finding indicated that multiple events, such as cell interactions, may influence the limiting dilution results. Similar data were obtained in eight other patients with AML. In all, a difference in limiting dilution was observed between cultures with growth medium alone, compared to cultures with either LCM or PHA. The minimum cell number to yield 50\% positive cultures varied between 3 x 10^2 and 1.5 x 10^4. For individual cell populations, the difference in limiting dilution between cultures in growth medium and cultures with added stimulation varied from 3- to 20-fold.

A difference between results for cultures with and without added LCM can also be shown by plotting cell concentration against ^3HTdR incorporation on a log:log scale. Figure 3 is such a plot for data from patient R. The difference is evident between cultures containing growth medium alone and those with added LCM. In addition, for cultures with incorporation in excess of 10^3 counts/min, the slope of the line relating cell number and ^3HTdR incorporation is greater for cultures in growth medium alone than for cultures supplemented with LCM. This convergence of results at higher cell numbers explains the failure to observe LCM stimulation in kinetic experiments using 2 x 10^3 cells/ml.
Fig. 4. Results of two experiments using cells from a patient, MAG, with acute myeloblastic leukemia. Data shown as a log-log plot of cell concentration versus counts/min/culture. , cultures in growth medium alone; , growth medium alone with \(10^3\) irradiated cells; , growth medium supplemented with leukocyte conditioned medium; , growth medium supplemented with \(10^5\) irradiated cells and \(0.5\%\) PHA; , growth medium and \(0.5\%\) PHA.

Qualitatively similar data were obtained with cells from the other leukemic patients, although patient-to-patient variation of approximately 50-fold was observed in the number of cells required to yield incorporation above \(10^3\) counts/min.

Mixing Experiments

Cells from a patient with AML (MAG) were used in mixing experiments designed to demonstrate cellular interactions. Preliminary kinetic experiments demonstrated a peak increased incorporation of \(^3\)HTdR on day 8 and modest stimulation with either LCM or PHA. Figure 4 contains the data from two experiments in which varying numbers of MAG cells were cultured for 8 days.
under the following conditions: (1) growth medium alone, (2) growth medium plus 10^5 irradiated cells, (3) 0.5% PHA and growth medium, (4) 15% LCM and growth medium, and (5) 10^5 irradiated cells plus PHA in growth medium. 

\(^3\)HTdR incorporation increased with increasing cell number for values in excess of 10^3 counts/min/culture. Marked stimulation was observed when LCM was added to cultures; an equivalent or greater stimulation was seen when 10^5 irradiated cells with PHA were added. Irradiated cells alone did not affect incorporation. In the presence of PHA alone, incorporation increased rapidly for cell numbers between 10^4 and 10^5 cells/ml. At the latter cell concentration, \(^3\)HTdR incorporation in PHA-stimulated cultures approached that found in cultures containing irradiated cells and PHA.

The stimulatory effect of irradiated autologous cells and PHA on \(^3\)HTdR incorporation by a constant small number (2 × 10^4) of intact cells was found to be related to the number of irradiated cells added. Data from one of two similar experiments are shown in Fig. 5. \(^3\)HTdR incorporation increased approximately linearly with added irradiated cells in the presence of PHA, to a value nearly twice that observed in the presence of LCM alone. Control cultures containing growth medium alone, irradiated cells without PHA, or PHA alone showed low levels of incorporation. The results of these cell-mixing experiments provided quantitative evidence for cellular interactions between an irradiated PHA-stimulated subpopulation and a subpopulation capable of \(^3\)HTdR incorporation.

Control Studies on Peripheral Leukocytes From Normal Subjects

Leukocytes from normal human subjects were studied in experiments of designs similar to those described above. Kinetic data for PHA-stimulated cultures are presented in Fig. 6. Under the conditions used for leukemic cells

![Fig. 6. Kinetics of \(^3\)HTdR incorporation by normal leukocytes in the presence of PHA.](image-url)
Fig. 7. Log-log plot of normal leukocyte cell number against \(^3\)HTdR incorporation in the presence of growth medium alone, two preparations of leukocyte conditioned media, 0.5% PHA, and \(10^5\) irradiated autologous cells plus PHA.

(2 \(\times\) \(10^5\) cells/ml), a peak increase in \(^3\)HTdR incorporation was observed on day 3 and maintained at or near the peak value until day 10. It was feasible, therefore, to investigate the effect of cell number on \(^3\)HTdR incorporation using the same experimental design applied to leukemic leukocytes.

Figure 6 also contains kinetic data for cultures at a cell concentration of \(10^6\) cells/ml, a value more usual for studies of normal leukocytes stimulated by PHA. Under these conditions, the usual peak between days 3 and 4 was observed with a rapid falloff thereafter.

The results of a representative cell-mixing experiment are shown in Fig. 7. Significant \(^3\)HTdR incorporation was not observed in cultures with growth medium alone. Upon addition of either of two preparations of LCM, incorporation increased with increasing cell number. In the presence of PHA alone, incorporation increased rapidly with increasing cell number, reaching values in excess of those for LCM-containing cultures. At low cell numbers, cultures containing \(10^5\) irradiated autologous leukocytes and PHA incorporated ten times the amount of \(^3\)HTdR as cultures with PHA alone. With increasing numbers of intact cells, \(^3\)HTdR incorporation in cultures with irradiated cells plus PHA increased, reaching values equivalent to those in cultures with PHA alone at intact cell concentrations of approximately \(3 \times 10^5\)/ml.

As in the case of leukemic leukocytes, the effect of added irradiated cells and PHA on \(^3\)HTdR incorporation by normal leukocytes depended quantitatively on the number of irradiated cells. Data from a single experiment are shown in Fig. 8. \(^3\)HTdR incorporation by a fixed number (4 \(\times\) \(10^4\) cells/ml) of normal leukocytes was observed as the number of irradiated autologous cells was increased in the presence of PHA, and approached the value obtained for \(10^5\) nonirradiated cells/ml cultured with PHA alone. Control cultures contain-
Radiation Sensitivity of $^3$HTdR Incorporation by Cells From Leukemic Patients and Normal Subjects

In previous experiments, radiation survival data for cells from leukemic blood were consistent with cellular proliferation as a basis for increased $^3$HTdR incorporation as a function of time in culture. In the present series, survival curve data were obtained for the peripheral cells of five patients with AML and one with CML. Cells were irradiated with increasing doses using a $^{137}$Cs irradiator and immediately diluted to appropriate cell number in either growth medium or growth medium with PHA or LCM (see Materials and Methods). The cultures were incubated for the time required to reach peak $^3$HTdR incorporation as indicated by kinetic experiments. They were then pulsed with $^3$HTdR, and survival was expressed as percentage of control unirradiated cultures. Values for $D_0$ varied from 110 rads to 180 rads and for the extrapolation number ($n$) from 1 to 6. Data in these ranges were obtained for cells cultured in growth medium alone or in the presence of either LCM or PHA.

Similar studies on normal leukocytes yielded different results. Figure 9 depicts radiation survival curves from two experiments on normal leukocytes cultured with $0.5\% \text{ PHA}$, and for comparison, two experiments on leukemic cells (patient MAG) carried out under the same conditions of stimulation, incubation time, and cell number. The survival curve for leukemic cells was typical of those reported for effects of radiation on cell proliferation and for
various mammalian cells, with an initial shoulder \((n = 5)\) followed by an exponential decrease with a \(D_0\) of 120 rads. In contrast, for normal leukocytes, the initial shoulder was much more pronounced; little decrease in survival was observed for doses under 400 rads.

Although the parameters for normal leukocytes are still compatible with cellular proliferation as a basis for increased \(^3\)HTdR incorporation, they are atypical and differ from the survival curve parameters obtained for leukemic leukocytes. The survival data for normal leukocytes are consistent with those of Rickinson and Ibery, who irradiated cultures at a density of \(10^6\) cells/ml and pulsed with \(^3\)HTdR after a maximum of 72 hr. At doses greater than 500 rads, these authors have observed a decreased radiation sensitivity, unrelated to proliferation. Their control data make artifacts related to intracellular thymidine pools unlikely. However, in the present context, radiation has been used to compare the behavior of normal and leukemic leukocytes and to support a proliferative basis of \(^3\)HTdR incorporation for the latter.

**DISCUSSION**

The results presented above have provided further support for the view that leukemic peripheral blood contains interacting subpopulations of cells capable of proliferation and of cells capable of modulating that proliferation. Under conditions of limiting dilution, a minority population of proliferating cells could be detected. In the presence of growth medium alone, the dilution end point for \(^3\)HTdR incorporation occurred at a larger cell number per culture than when leukocyte-conditioned medium (LCM) was added, as expected if the stimulatory subpopulation was the limiting factor under the former culture conditions but not in the latter. Even in the presence of LCM, where the limiting factor was presumably the size of the proliferating population, the detectable proliferating population was only a small minority (less than 1\(^\circ\)) of the total population. The majority of the population, consisting mainly of blast cells, apparently did not participate in any major way in these cellular interactions.

Mixing experiments, in which irradiated, PHA-treated stimulatory cells were added in varying numbers to cultures containing a fixed number of autologous
responder cells, provided evidence for a quantitative relationship between the number of stimulatory cells added and the increase in $^3$HTdR incorporation seen in the responding population. This relationship could provide the basis for a quantitative assay for the stimulatory cells that could be used to investigate their properties. For example, the present experiments did not distinguish between the possibility that the stimulator and responder cells were members of distinct cell classes, or if they merely reflected different functional properties of a single class of cells. However, the partial physical separation of stimulatory and responding cells described previously for cells from a leukemic population supports the view that the interaction involves two distinguishable cell classes.

Stimulation of $^3$HTdR incorporation in cultures of leukemic leukocytes was achieved in three ways: by addition of leukocyte-conditioned medium prepared in the presence of PHA, by the direct addition of PHA to the cultures, especially to cultures containing relatively large numbers of cells, and by the addition of irradiated (1000 rads) autologous leukocytes in the presence of PHA. Addition of irradiated cells in the absence of PHA did not increase $^3$HTdR incorporation; while in the presence of a fixed concentration of PHA, the amount of stimulation was directly proportional to the number of irradiated cells added. These results supported the view that PHA acted indirectly through a nonproliferating cell population, present in rate-limiting numbers in leukemic cell populations and able to produce stimulatory factors. This stimulatory activity present in PHA-treated cultures of leukemic leukocytes appeared to be at least partially separable from the four molecular species known to stimulate granulopoietic colony formation in culture.\textsuperscript{16,17} In preliminary experiments,\textsuperscript{18} the latter colony-stimulatory activity was absorbed more readily to hydroxylapatite than was the activity able to stimulate $^3$HTdR incorporation by leukemic leukocytes.

Similar studies of the effect of cell number on $^3$HTdR incorporation in cells from the blood of normal subjects were also compatible with cell-cell interaction. The most notable finding was that, as in the leukemic case, the addition of irradiated leukocytes with PHA to small numbers of intact autologous cells resulted in a ten-fold increase in $^3$HTdR incorporation. Less stimulation was observed with LCM, and little or no $^3$HTdR incorporation occurred in the absence of stimulator over the range of cell numbers tested. These observations were reminiscent of those of Lohrmann et al.\textsuperscript{19} who demonstrated that DNA synthesis could be stimulated in separated B lymphocytes by the addition of T cells with a variety of mitogens, provided that monocytes were also present. They postulated that in addition to a direct stimulation of T cells by mitogen, an indirect, monocyte-dependent enhancement occurred. As a basis for the latter, they postulated “mitogen processing” by monocytes. It seems equally plausible that soluble substances were involved, as suggested by Gery et al.\textsuperscript{20} and Andersson et al.\textsuperscript{21} Both groups reported soluble factors released by T cells.

It is not clear from the results reported here that the cellular interactions observed in leukocytes from normal subjects differed qualitatively from those seen in leukemic populations. Quantitative differences were seen. For example,
a subpopulation in leukemic peripheral blood was also able to incorporate $^3$HTdR in cultures containing growth medium alone, while few if any normal leukocytes were able to do so under these culture conditions. Also, the radiation survival curves differed for the leukemic and normal leukocytes. These curves were constructed to probe the cellular basis of increased $^3$HTdR incorporation. Radiation survival curve parameters within the range of those of mammalian cells obtained using assays based on colony formation have been considered evidence for a proliferative basis. For leukemic populations, the survival curve parameters were typical of those associated with proliferation, and provided strong support for proliferation as the basis of the observed increase in $^3$HTdR incorporation by leukemic cells. For normal leukocytes, the radiation survival curve parameters were different, but not so atypical as to rule out a contribution of proliferation to $^3$HTdR incorporation by normal leukocytes after 7 days incubation with PHA.

The curves for normal and leukemic leukocytes differ most markedly in the initial shoulder portion of the curves. This portion of a survival curve reflects the accumulation of sublethal radiation damage, and the extent of the shoulder portion of the curve is usually characteristic of the cell type involved. The difference in survival curve parameters provides the strongest evidence presently available that the major cell type detected in leukemic populations on the basis of its capacity to incorporate $^3$HTdR is not identical with the one detected in normal populations. This view is supported by previous experiments in which chromosome markers characteristic of a leukemic population have been found in cells harvested from cultures at the time of maximum $^3$HTdR incorporation. These results do not rule out the possibility that a proliferative subpopulation of cells in leukemic peripheral blood may have properties indistinguishable from the proliferative population in normal peripheral blood. Cell-culture techniques for the detection of such cells and for distinguishing unequivocally between normal and leukemic clones are not yet available.

Some of the complexities of these interacting subpopulations in leukemic and normal peripheral blood might be resolved more readily if colony assays were available for the proliferative components. Recently, Dicke et al. have reported colony formation in soft agar cultures of leukemic marrow cells, where the cells were first exposed to PHA in suspension, and subsequently cultured over a hard agar underlay. These authors provided evidence that cells in such colonies were of leukemic origin. The requirement for PHA stimulation, observed by Dicke et al., provided a link with the experiments reported in this paper. In preliminary work, we have observed colonies in methylcellulose cultures of leukemic peripheral leukocytes from two of the patients (MAG and R) reported in the present paper. Such colony formation was stimulated either by leukocyte-conditioned medium prepared in the presence of PHA or by semipurified preparations capable of stimulating $^3$HTdR incorporation in liquid cultures but not granulopoietic colony formation. Under these culture conditions, PHA by itself did not stimulate colony formation by leukemic peripheral leukocytes. Studies designed to delineate the relationship between the colony formation reported by Dicke et al. and $^3$HTdR incorporation in liquid culture,
and the LCM-stimulated colony formation we have observed may contribute to an understanding of regulatory mechanisms that exist in normal hematopoiesis and any aspects of such regulatory mechanisms that persist in leukemia.

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**REFERENCES**


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