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SENSITIVITY OF HUMAN LEUKEMIC CELLS TO ANTI-CANCER AGENTS IN CULTURE*

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Abstract—Special-conditioned medium prepared from overgrown cultures of murine leukemic cells was found to support short-term reproduction (24–96 hr) of human leukemic cells in culture. Failure of three preparations to grow in culture may be due to the instability of the special medium when stored for more than 5 days. Leukemic cell preparations from 12 patients had a variable growth response *in vitro* and also exhibited a different and characteristic sensitivity to anti-cancer agents. The selection of specific anti-cancer agents for treatment of leukemic patients may be possible with the development of this drug-sensitivity test *in vitro*.

IT HAS BEEN reported by Pluznik and Sachs[1] and Bradley and Metcalf[2] that the growth of a fraction of murine hematopoietic cells *in vitro* occurs in the presence of a stimulating factor. Substances which stimulate and enhance the growth of murine or human bone marrow cells include cell feeder layers,[1,3,4] urine,[5] serum,[6] conditioned medium,[7,8] spleen extract[9] and a supernatant from human embryo kidney cells.[10]

Leukocyte cultures from peripheral blood obtained from patients with leukemias and lymphomas have been established when various media were used.[11] The ability of human bone marrow to form myeloid colonies in soft-agar has been investigated and the relationship between normal and leukemic cell lines studied.[12] In addition, cell differentiation *in vitro* may be regulated by an inducing factor.[13]

This study concerns: (1) the development of a new medium which supports the immediate and short-term reproduction in culture of human leukemic cells and (2) the sensitivity of human leukemic cells *in vitro* to certain anti-cancer agents.

MATERIALS AND METHODS

Peripheral blood and bone marrow aspiration specimens from leukemia patients with greater than 50% blast cells or greater than 50% lymphocytes were obtained in plastic syringes containing 0·3 ml sterile heparin (1000 units/ml), transferred to 16 × 125 mm glass screw-cap culture tubes with a rubber seal and allowed to sediment at 4° for 2 hr. After centrifugation at 600 *g* for 5 min at approximately 25°, the

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buffy coats were removed by careful aspiration and the cells were resuspended in Fischer's medium (FM) supplemented with 10% horse serum (FMS). Cell concentration should not exceed 5×10^7/ml. Cells were immediately diluted to a final concentration of $1–2 \times 10^5$/ml in FMS plus 20% special-conditioned medium (SCM) in a 500-ml glass bottle. Five-ml aliquots were then distributed in culture tubes (16 × 125 mm glass screw-cap tubes with a rubber seal) to which a dilution series of different anti-cancer agents were added. Three replicates of each incubation were counted daily with a Coulter Counter (model B) for a period of time ranging from 2 to 4 days. The time from removal of specimens from the patient to incubation at 37° was minimized and did not exceed 4 hr.

The special-conditioned medium was prepared from mass cultures of L5178Y murine leukemic cells in FMS. Cells for the inoculum were obtained from cultures grown in FMS in the exponential phase of growth. The cells were maintained at 37° throughout the entire procedure and diluted in the prewarmed FMS (37°) to a final concentration of 1×10^5/ml in a volume of 40 ml in a 140-ml glass flask with a rubber-lined screw cap. Cells were incubated at 37° for 72 hr. The doubling time of the cells is $12 \pm 1{\cdot}5$ hr. Such overgrown culture provided an optimum source of this medium. The medium was harvested by centrifugation at 800 *g* for 5 min at approximately 25°, sterilized by filtration through a HA Millipore filter (0·45 μm) and immediately stored in aliquots at −20° and used within a time period of 5 days after freezing.

Horse serum (Grand Island Biological Co., Grand Island, N.Y.) was assayed using murine leukemic cells (L5178Y): (1) for growth-promoting activity by outgrowth method;[14] one unit of serum is defined as that level which permits the reproduction of one-half the cell doublings maximally obtained. Acceptable lots of serum have a unit activity of less than 3 per cent; and (2) for ability to promote colony formation by the soft-agar cloning technique;[15] acceptable lots of serum have a cloning efficency of 60–85 per cent at high serum concentrations (15 per cent and perhaps 10 per cent) and a unit activity of 7 per cent or less. Serum is stored by the manufacturer in large quantities (20–40 liters) for a period of no longer than 3 months, received in small quantities, stored at −16° and used within a period of 3 weeks. Each assay is repeated in a separate experiment. Serum of known unit activity is used as a control.

Glassware used in cell culture work is prepared according to standard procedures.

Fischer's medium (10 × concentrate, Grand Island Biological Co., Grand Island, N.Y.) is diluted with sterile water to which dry sterile $NaHCO_3$ has been added. Medium (10 ×) is received and stored at 4° and used within a time period of 40 days. Horse serum of known unit activity is added to give a final concentration of 10 per cent in FM and a five-point growth curve obtained. The best fit, straight line is determined using a program and an Olivetti Computer. As the control, a prior preparation of FM is used. The 95 per cent confidence limit must fall within the range of 1·5 hr doubling time of the controls.

Drugs were prepared in sterile deionized, glass-distilled water and sterilized by filtration through a Swinny Type Millipore filter (0·45 μm). A dilution series of different anti-cancer drugs were added to the culture tubes in a volume of 0·05 ml for each drug, singly, simultaneously and sequentially and incubated at 37°.

Drugs used in the cell culture studies and in patients are selected in relation to

their known or potential therapeutic anti-leukemic efficacy and are summarized below:

6-Azauridine: NSC-32074; 6-AzUR, CalBiochem Co. 6-Azauridine triacetate: NSC-67239; Azaribine, AZ, CalBiochem Co. Chlorambucil: NSC-3008; Leukeran, Clb, Burroughs Wellcome & Co. Cystosine arabinoside: NSC-63878; cytosine, 1-β-D-arabinofuranosyl-, ara-C, Cytosar, The Upjohn Co. Cyclophosphamide*: NSC-26271; Cytoxan, CTX, Mead Johnson Laboratories. 5-Fluoro-2'-deoxyuridine: NSC-27640; 5-FUDR, Hoffman–La Roche, Inc. Methotrexate: NSC-740; MTX, Lederle Co. 6-Methylmercaptopurine riboside: NSC-40774; 6-MMPR, NCI, NIH. Prednisone: NSC-10023; Pred, The Upjohn Co. 6-Thioguanine: NSC-752; 6-TG, Burroughs Wellcome & Co. Vincristine: NSC-67574; Oncovin, VCR, Eli Lilly & Co.

Compounds are prepared, sterilized and diluted according to the methods described above.

RESULTS AND DISCUSSION

The correlation between drug sensitivity *in vitro* and responsiveness *in vivo* has been demonstrated in murine leukemic cell culture systems.[16] A test for assessing the sensitivity of human lymphocytes to chlorambucil *in vitro* has been reported.[17] Those human leukocyte cell lines which multiply indefinitely in culture presumably represent only a small fraction of the same cell populations present in the human host. Cloning efficiency of marrow cells from human or experimental animals range from 1×10^{-3} to 4×10^{-3}.[4,8,10,18]

A variety of novel special culture conditions have been investigated by us for their ability to support the immediate and short-term multiplication of various human leukemic cell preparations. Specimens were obtained from seven acute leukemia patients and one patient with chronic myelogenous leukemia (CML) in blastic crisis with greater than 50% blast cells and from four chronic lymphocytic leukemia (CLL) patients with greater than 50% lymphocytes. Peripheral blood was used primarily for these studies, but bone marrow aspirates were obtained when the peripheral white blood cell count (WBC) was low or showed less than 50% blast cells. Several patients had simultaneous peripheral blood and marrow aspirate studies. The development of SCM (Materials and Methods) was optimal for these initial studies. In all but three of the 14 cell preparations, 20% SCM was adequate to support short-term cell reproduction in culture.

The relationship between the concentration of SCM and growth of leukemic cells is illustrated in Fig. 1. With the concentration of 20% SCM, the maximum increase in cell growth over that of the non-conditioned medium (FMS) was obtained. The lower cell number found at the higher concentration of SCM may be due to the presence of an inhibitory substance. Therefore, 20% SCM in FMS was used in all subsequent drug-sensitivity studies.

In this study, growth is defined as an increase in cell number of the non-drug-treated control, drug-sensitivity as a decrease and drug-resistance as a constant or increase in number of drug-treated cells when compared to the inoculum. This arbitrary definition of sensitivity in culture cannot be evaluated due to the limited number of patients studied. Effectiveness of drug combinations was evaluated using

* Cyclophosphamide requires activation *in vivo* and cannot be studied in the culture system.

TABLE 1. GROWTH AND DRUG SENSITIVITY OF HUMAN LEUKEMIC CELLS TO ANTI-CANCER AGENTS IN CULTURE*

			Control			Drug sensitivity in culture (cells/ml × 10^{-4})										
Patient	Specimen	Date	Inoculum	Max. cells/ml	Total cells/time period	ara-C	6-TG	6-MMPR	Clb	VCR	Pred	MTX	AzUR	5-FUDR	MTX→ara-C	6-TG→6-MMPR
A. D.†	P	8/ 3/71	15·0	20·3	20·3/72 hr	3·4	5·6		8·7			8·4				
S. P.†	P	5/12/71	18·0	25·0	25·0/48 hr	15·0	14·0		13·2			14·0				
E. S.†	P	5/17/71	26·0	64·0	64·0/24 hr	46·0	44·0	38·0	52·0	46·0	48·0	46·0			38·0	
J. G.†	P	8/10/71	40·0	42·0	34·0/72 hr	13·0	29·0		22·0			29·3				
J. M.‡	M	3/17/71	17·0	20·0	20·0/48 hr	9·8	14·0	10·6		11·0		17·0	16·4			
B. B.§	M	1/24/71	8·0	9·5	8·1 48 hr					6·3	6·8					
F. C.‖	M	3.3/71	20·0	23·0	23·0/42 h	13·0						24·0				
L. G.¶	P	2/18/71	100·0	183·0	183·0/48 h	122·0						128·0				
D. S.¶	P	11/15/71	12·0	13·0	13·0/48 h	2·0	2·8	3·1		1·5		7·0				
G. M.¶	P	6/28/71	19·0	38·0	36·0/96 hr	2·4	2·0					26·0	19·0			
G. M.	M	1/24/72	14·0	19·0	16·0/48 hr	7·0	8·0				12·0	15·0			5·6	
G. M.	P	6/12/72	20·0	17·0	12·0/96 hr	3·0	3·0	6·0			11·3	12·0				2·4
A. S.¶	P	5/17/72	44·0	64·0	64·0/24 hr	50·0	32·0	50·0		44·0	46·0	46·0			41·0	
A. C.¶	M	6/10/72	13·0	18·0	18·0/48 hr	18·0	14·0	13·0		17·0	21·0	18·0		20·0	16·0	

* Leukemic cell preparations from patients were incubated at 37° with various anti-cancer agents. Cell number was determined from 24 to 96 hr by a Coulter Counter (model B). Column 4 is to be compared with column 5 for growth and with columns 7 through 17 for drug sensitivity. The concentration of agents used was 1×10^{-5} M except for Clb at 20 μg/ml or 4·2 μg/ml (S.P.), and where indicated in the text. Each observation is the mean value of three replicates. P, peripheral blood; M, bone marrow aspirate.

† CLL, chronic lymphocytic leukemia.

‡ CML, chronic myelogenous leukemia with acute blastic crisis.

§ ALL, acute lymphoblastic leukemia.

‖ AUL, acute undifferentiated leukemia.

¶ AGL, acute granulocytic leukemia.

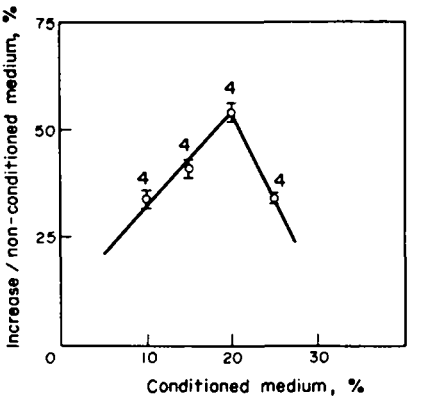

FIG. 1. Leukemic cell preparations from patients were incubated at 37° for 72 hr with different concentrations of special-conditioned medium (SCM) in Fischer's medium containing 10% horse serum. Cell number was determined by a Coulter Counter (model B) and the per cent increase over non-conditioned medium control (linear) was plotted against per cent SCM (linear). Each point is the mean value of four experiments with three replicates for each experiment. Vertical lines indicate ± one standard error of the mean.

the drugs singly, simultaneously and sequentially in certain culture studies. Morphological studies were performed in several patients.[19] The cells maintained their normal morphology at 48 and 72 hr in SCM controls.

Two patient culture studies will be discussed to demonstrate the growth curves and drug effects at 24-hr intervals. Some of the culture studies did not show growth throughout the entire incubation. Since the initial aim was to determine the efficacy of the growth medium, only four of the patients studied were treated on the basis of results *in vitro*.

Response of CLL cells from peripheral blood of patient A. D. to ara-C and 6-TG is shown in Table 1. A growth curve (Fig. 2) showed that the cell number in the non-

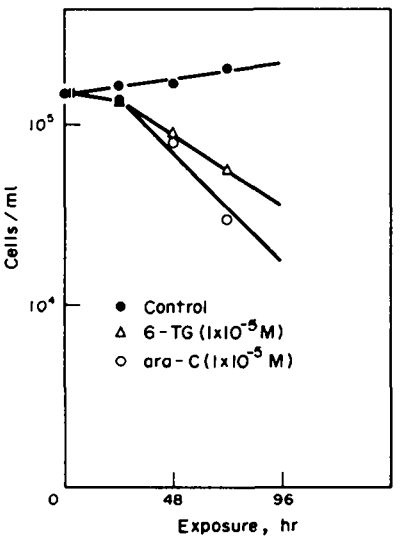

FIG. 2. Chronic lymphocytic leukemic cell preparation ($1{\cdot}5 \times 10^5$/ml) was incubated with 6-TG (1×10^{-5} M) and ara-C (1×10^{-5} M) at 37° for 72 hr. Cells were counted daily with a Coulter Counter (model B). Each point is the mean value of three replicates.

treated control increased approximately 10 per cent every 24 hr for the 72-hr period studied. After 24 hr of incubation, ara-C exhibited greater inhibitory effect than 6-TG at equimolar concentrations ($1{\cdot}0 \times 10^{-5}$ M). With ara-C the cell number decreased by 50 per cent per 24 hr compared to 36 per cent per 24 hr with 6-TG. Chlorambucil at 20 μg/ml, the highest concentration used, was less inhibitory (Fig. 3) than ara-C or 6-TG at $1{\cdot}0 \times 10^{-5}$ M. Specific anti-leukemic therapy was not indicated in this patient at the time of this study.

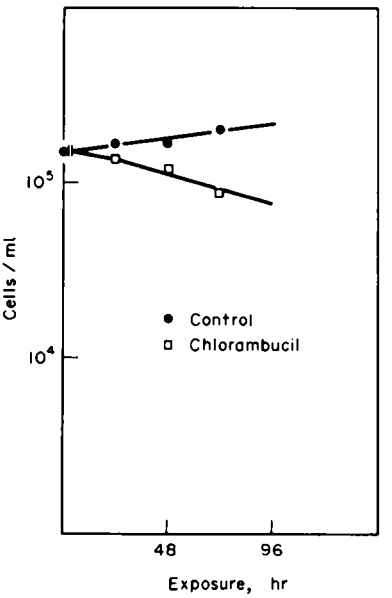

FIG. 3. Chronic lymphocytic leukemic cell preparation ($1{\cdot}5 \times 10^5$/ml) was incubated with chlorambucil (20 μg/ml) at 37° for 72 hr. Cells were counted daily with a Coulter Counter (model B). Each point is the mean value of three replicates.

Responses of acute granulocytic leukemia (AGL) cells from patient G. M. to ara-C, 6-TG, MTX, 6-MMPR and Pred, studied at different times during the course of disease, are shown in Table 1. Cells obtained at the time of diagnosis were sensitive to both ara-C (1×10^{-5} M) and 6-TG (1×10^{-5} M). The rate of decrease in cell number was approximately 50 per cent per 24 hr with both of these agents. The increase in cell number in the non-treated control was approximately 50 per cent in the first 24 hr, then decreased very slightly during the subsequent time period. The cells were not sensitive to a very high concentration of MTX ($1{\cdot}0 \times 10^{-5}$ M) (Fig. 4). The patient received several chemotherapy regimens selected independently of the findings *in vitro*.

Seven months after the initial study, a bone marrow aspirate was used for the sensitivity study *in vitro* due to the low peripheral WBC. The response to ara-C, 6-TG and 6-MMPR in culture was much less than that observed prior to the initial induction course of anti-leukemic therapy. The decrease in cell number at the end of 48 hr was 50 per cent for both ara-C and 6-MMPR and 44 per cent for 6-TG. The cells were not sensitive to Pred. MTX was not inhibitory, but when cells were

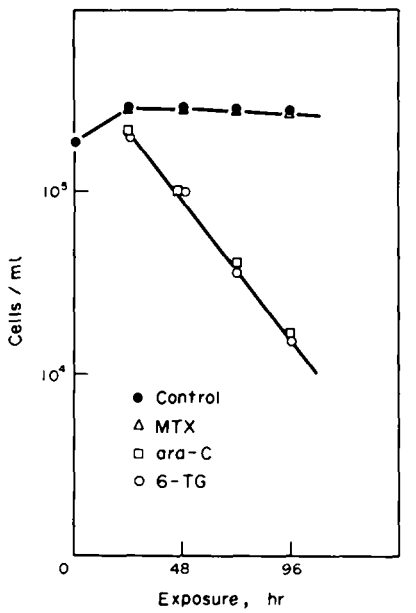

FIG. 4. Acute granulocytic leukemic cell preparation ($1{\cdot}9 \times 10^5$/ml) was incubated with ara-C (1×10^{-5} M), 6-TG (1×10^{-5} M) and MTX (1×10^{-5} M) at 37° for 96 hr. Cells were counted daily with a Coulter Counter (model B). Each point is the mean value of three replicates.

pretreated with MTX for 1 hr and followed by ara-C, there was a slightly greater decrease in cell number than that observed with ara-C alone (Table 1). The patient was subsequently treated with sequential MTX and ara-C and showed a significant decrease in the bone marrow blast cells from 50 to 10 per cent.

Approximately 1 yr after the initial study, peripheral blood was used for the sensitivity study *in vitro*, since patient G. M. had a WBC of 19,700, mostly leukemic cells (70% blast cells). The cells in the non-treated control decreased slightly over a period of 72 hr, approximately 10 per cent per 24 hr. This resulted when the SCM used for this patient had been stored at −20° for over 120 hr. In two previous studies, cells from this patient demonstrated significant reproduction in culture (Table 1).

These studies undertaken during different phases of disease in patient G. M. indicated that: (1) the sensitivity of the cells in culture to anti-cancer agents, ara-C and 6-TG in this case, decreased during treatment of the disease, possibly due to the disease itself or change in drug sensitivity; (2) although MTX given alone was not inhibitory, it may have enhanced the killing effect of ara-C when cells were pretreated with MTX.[20,21]

Responses of AGL cells from patient A. S. to various anti-cancer agents are shown in Table 1. Cells were only sensitive to 6-TG. In this patient the early selection of 6-TG based on drug-sensitivity studies *in vitro* resulted in a rapid complete remission. This avoided the necessity of utilizing a multiple drug regimen which might have been ineffective with the hazard of drug-induced host toxicity. It is known that multiple drug regimens may offer both increased rates of induction remission and increased duration of remission. However, there are approximately 30–50 per cent of patients with AGL who fail to respond to induction therapy with present multiple

drug regimens. It is possible that selection of a single sensitive agent may be the most effective therapy for certain individuals; however, one must continue to study drug combinations to achieve optimum therapeutic benefits.

The leukemic cells obtained from the bone marrow aspirate of patient A. C. were not sensitive to any of the drugs used in culture studies (Table 1). He failed to respond to therapy including the drug ara-C which is considered the agent of choice for AGL. However, possible initial drug-resistance in culture and in the patient was suggested in this study.

Leukemic cell preparations from 12 patients have been studied. Each individual leukemic cell population has shown a characteristic sensitivity which differs when compared with the sensitivity in culture of other leukemic cell populations including those of similar histological types (Table 1). In all but three preparations, 20% SCM was adequate to support reproduction. The preparations failed to grow in the SCM after 24 hr of incubation, possibly because these studies were performed when the SCM used had been stored at $-20°$ for over 120 hr.

It was evident from our studies that no single agent was effective in all leukemic cell cultures including those of similar histological types. This significant finding indicates the necessity to select drugs for treatment on an individual basis. Results in the few patients studied initially have shown clinical correlation, and it is suggested that a more extensive trial is warranted to evaluate this method. It is hoped that the specific drug-sensitivity test of leukemic cells in culture made possible by the development of the novel special-conditioned growth medium will permit a rational approach to the selection of specific drugs for the treatment of each individual leukemic patient and provide optimal therapeutic benefits. This system may eventually offer significant advantages for the selection of anti-cancer agents.

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