Tissue Culture of Primitive Human Myeloid Cells for the Study of Cellular Proliferation

Preliminary report on a growth inhibitor in the heated serum of two patients with chronic myelogenous leukemia (CML) following splenic irradiation

By Stephen B. Shohet and Frank H. Gardner

Several investigators have noted the phenomenon of suppression of hemopoiesis by splenic irradiation. This phenomenon as applied to chronic myelogenous leukemia (CML) was given the name of "abscope effect" by Mole in 1953. Recently Maurice attempted to demonstrate this effect under controlled conditions using blood from the irradiated spleens of intact healthy rabbits. He was able to show that such blood would depress the white count, the reticulocyte count and marrow mitoses in homologous rabbits. Previously Li had been able to demonstrate that the plasma taken from patients undergoing splenic irradiation could subsequently depress the white count of these same patients when it was administered at a later date. These studies suggest the possibility that a humoral substance might be liberated from the irradiated organ which could depress hematopoiesis.

The establishment of a stable line of cultured cells (64:10) derived from the blood of a patient with acute myelogenous leukemia and its recent adaptation to growth in a tissue culture medium which requires human serum for growth has made it possible to investigate such a humoral factor by substitution of the serum of irradiated patients for normal serum in the medium. The purpose of this study is to present the results of the incubation of these cells in media containing the serum of two patients with CML in whom a fall in the peripheral white blood cell count had been observed following splenic irradiation.

Materials and Methods

Modified McCoy's medium was used for all cultures and cell suspensions. Cells were prepared from vigorously growing stock cultures by gentle centrifugation and resuspension in fresh media. Innocula were planted into the various test media at concentrations of.

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This study was partially supported by U.S. Public Health Service Grant Number AM-00965. Part of this work was performed while one of us (SBS) was a Clinical Associate at the National Cancer Institute, Laboratory of Clinical Pharmacology.

First submitted March 28, 1967; accepted for publication August 4, 1967.

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4 \times 10^4 \text{ cells/cc}. \text{ These cultures were then statically incubated in 4 oz. soft glass closed bottles under an atmosphere of 5 percent CO}_2 \text{ in air. Cell counts were made from aliquots of cell suspensions in duplicate on a Model B Coulter Counter. These counts were checked against hand counts at frequent intervals and found to agree within 8 percent. Four flasks were counted daily in all experiments. The variation is indicated by brackets on all of the graphs of data. All culture bottles were briskly shaken to disburse clumped cells before counting. When visually counted at this stage, more than 94 percent of the cells were present as single cells. Rare doublets and triplets were responsible for the residual 6 percent.}

The patients with CML were in the midst of a course of daily 100 r irradiation to a circumscribed splenic portal at the time of these studies. The irradiating source was a 6 mev linear accelerator. 100 rads were delivered at a 100 cm. target distance through a lead filter with a half value of 12.5 mm. A third patient with metastatic carcinoma of the breast who was also in the midst of a course of radiotherapy received 272 rads to her left superior thorax one hour prior to blood sampling. The source, target distance, and filtration were the same as above.

Samples of blood were collected either 1 or 6 hours after irradiation. The blood was passed through a calcium-sodium ion exchange column to prevent clotting.\textsuperscript{*} \text{ The blood was then centrifuged at 800 g at 4 C. for 45 minutes in an International Centrifuge with a swinging bucket head. The plasma was then separated and recalcified with 5 meq./l. of sterile calcium chloride. The resultant serum was then heated at 55 C. for 30 minutes to remove suspected non-specific growth inhibitors\textsuperscript{11-13} and to facilitate clot retraction.}

These sera were then substituted for, or mixed with, normal serum in the tissue culture media. The various serum mixtures on cell growth were then assayed by doing daily cell counts on quadruplicate flasks. In general, the serum was used immediately after processing, but on one occasion it was frozen at -80 C. for 3 days, rapidly thawed to 37 C. with constant agitation and then used directly.

\textsuperscript{*}Bag #F1633 Fenwall Labs., Morton Grove, Illinois.
Fig. 2.—Cell growth in various concentrations of the serum of patient number one taken following irradiation; normal serum used as diluent.

Fig. 3.—Cell growth in various concentrations of the serum of patient number two taken following splenic irradiation; normal serum used as diluent.

Patients Studied
Patient 1 (V.M.) was a 62-year-old white female with recently diagnosed CML. Diagnosis was established by a white count of $1.3 \times 10^8$ with 40 percent myelocytes and 8 percent myeloblasts. Additionally, the spleen was palpable 12 cms. below the left midcostal margin and the bone marrow showed marked myeloid hyperplasia. The peripheral blood and
I HOUR POST SPLENIK IRRADIATION SERUM STORED AT -80 FOR 3 DAYS (SS)

Fig. 4.—Cell growth in the serum of patient number two taken one hour after splenic irradiation and then stored at -80 C. for three days prior to study.

Fig. 5.—Cell growth in the serum of patient number two taken after splenic irradiation and subsequently dialyzed against normal human serum.

BLOOD marrow were both positive for the Philadelphia chromosome by the method of Moorehead. Alkaline phosphatase levels were below 10 by the method of Ruttenberg.

Patient 2 (A.W.) was a 49-year-old white male with a diagnosis of CML known for one year. He presented at the time of the study with anemia. The spleen edge was palpable 16 cms. below the left costal margin. The white count was 100,000 per cubic mm. The differential showed 33 percent basophilic myelocytes, 10 percent myeloblasts, 20 percent mature basophils and 40 percent band and later neutrophils. His peripheral blood also was positive for the Philadelphia chromosome.
Fig. 6.—Cell growth in the serum of patient number two taken six hours after splenic irradiation.

Fig. 7.—Cell growth in the serum of patient number two taken after irradiation when WBC count and spleen size was no longer changing.

The nonleukemic patient was a 48-year-old white female studied four weeks following radical mastectomy for adenocarcinoma of the breast. At the time of study her peripheral white blood cell count was 9,000/cmm. with a normal differential.

Results

In general, a marked depression of the cell growth rate was found when serum obtained from the CML patients immediately after irradiation was used
in the tissue culture system. The same scale and coordinates are used throughout for all of the data presented in the figures. Cell number is plotted against the days of incubation.

Figure 1 shows the control pattern of growth of the cultured cells in the pre-irradiation sera from both patients. After approximately 24 hours of delay, the cells enter a period of rapid growth for approximately 3 days. Eventually cell crowding occurs and the growth rate declines. Figures 2 and 3 show the depressed growth rate of cultures in media containing up to 30 percent serum obtained within one hour after irradiation. Dilution of the freshly irradiated CML serum with normal similarly prepared serum tended to correct but did not eliminate this depressive effect in both cases. This dose related effect was still apparent with as little as 5 percent irradiated serum.

Figure 4 shows that frozen storage of the serum of patient number two at -80°C for three days did not reduce its potency as a depressor of cell growth.

Figure 5 similarly shows that the inhibitory factor was not removed by dialysis for 14 hours against normal human serum at 4°C.

Samples of serum obtained from the second patient 6 hours after irradiation did not depress the growth rate of cells (Figure 6). In addition, Figure 7 shows a remarkable reduction in the presence of inhibitor in the serum of this leukemic patient at a later stage in the course of his irradiation when his response to irradiation had dwindled to such an extent that his white blood count was stable at approximately 20,000 cells/cu. mm. and his spleen, though still large, was no longer shrinking. At this juncture, the growth of cells was barely depressed by his post-irradiation serum (compared with Figure 3).

Irradiation of a nonsplenic area did not produce a serum inhibitor (Figure 8). Here it is seen that serum from a patient with metastatic carcinoma of the
breast obtained following irradiation over the thorax did not depress the growth of the cultures.

**Discussion**

The growth of a stable cell line derived from the blood of a patient with acute myelogenous leukemia was inhibited by sera obtained from two patients with chronic myelogenous leukemia within 1 hour of splenic irradiation. The fact that this effect was noted only shortly after irradiation at first suggested that irradiation of the blood passing through the spleen might have generated free radicals capable of inhibiting cellular growth by direct oxidation of S-H enzymes or disruption of chromosomes. However, the demonstration of the effect after prolonged storage of frozen serum and the absence of the effect in the irradiated breast cancer control mitigates against any direct effect of short-lived free radicals.

The work of Perry and Marsh suggests one possible explanation as to the nature and mechanism of this inhibitor. These authors found that homogenates of both normal and chronic myelogenous leukemia (CML) leukocytes inhibit the in vitro uptake of thymidine H3 by CML cells whereas intact CML cells stimulate such incorporation. They suggested that the mechanism of this inhibitor may be related to a conversion of media thymidine to thymine by a phosphorolase in the homogenate. While they point out that the thymidine is "not thought to be in the normal intracellular pathway for DNA synthesis . . . the suggestion has been made that the enzymes involved in thymidine metabolism may be important in initiating and regulating DNA synthesis." This then would suggest that cells in the spleen might liberate a thymidine phosphorolase after massive lysis by irradiation. This enzyme then might influence cell division in distant cells by controlling levels of thymidine or other enzymes involved in its metabolism.

This may suggest a mechanism for feedback control of white cell levels by white cell lysis products. Such a mechanism combined with the purported long life of the intact young CML cell or the apparent tendency of CML cells not to enter with local tissue reaction where they would be liable to destruction might then explain the absence of normal marrow inhibition in this disease. Such an hypothesis is supported by the observations that inhibition of the test cells in our studies was noted only when presumably large numbers of cells were being destroyed during the time that the splenic mass was being reduced by irradiation.

**Summary**

Sera from two patients with chronic myelogenous leukemia obtained within one hour after irradiation of the spleen inhibited the growth of a stable cell line derived from the blood of a patient with acute myelogenous leukemia. This inhibitory factor was stable in vitro for one half hour at 55 C. and for 3 days at −80 C.

Serum from a patient with metastatic carcinoma of the breast following thoracic irradiation did not have a similar effect.
SUMMARIO IN INTERLINGUA

Seros ab duo patientes con chronic leucemia myelogene, obtenite intra un hora post ir-
radiation del splen, inhibiva le crescentia de un stabile linea de cellulas obtenite ab le san-
guine de un patiente con acute leucemia myelogene. Le factor de inhibition esseva stabile
in vitro durante un medic hora a 55 C. e durante 3 dies a —80 C.

Sero ab un patiente con carcinoma metastatic del mammas post irradiation non habeva
le mesme effecto.

ACKNOWLEDGMENTS

The authors are grateful to Dr. James T. Grace of Roswell Park Memorial Institute for
generously supplying the original cell strain. We are also indebted to Dr. William C.
Mohler of the National Cancer Institute for advice and encouragement in the early stages
of adapting and characterizing these cells.

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