[CANCER RESEARCH 40, 1062-1065, April 1980]
0008-5472/80/0040-0000$02.00

Growth Suppression of Human Leukemic Cells *in Vitro* by L-Ascorbic Acid[1]

Chan H. Park,[2] Mammo Amare, Michael A. Savin, and Barth Hoogstraten

Department of Medicine, University of Kansas Medical Center, Kansas City, Kansas 66103

ABSTRACT

The suppressive effect of L-ascorbic acid on the growth of bone marrow cells from patients with acute nonlymphocytic leukemia was studied using a modified agar culture method featuring daily feeding to allow the growth of leukemic cell colonies. In seven of 28 patients (25%), the numbers of leukemic cell colonies grown in culture were reduced to 21% of control by the addition of L-ascorbic acid (0.3 mM) to the culture medium. Glutathione did not suppress leukemic cell colonies although it has a similar oxidation-reduction potential to that of L-ascorbic acid. The addition of L-ascorbic acid reduced the pH of the medium. However, a comparable reduction of pH by the addition of HCl did not suppress leukemic cell colonies. In simultaneous cultures for leukemic and normal marrow cells, the suppression of leukemic cell colony was noted with a concentration of L-ascorbic acid as low as 0.1 mM (a concentration achievable *in vivo*), but normal myeloid colonies were not suppressed until the concentration of L-ascorbic acid reached an extremely high level (1 mM). In conclusion, growth of leukemic cells in culture was suppressed by L-ascorbic acid in a substantial proportion of patients with acute nonlymphocytic leukemia. This suppression was a specific effect of L-ascorbic acid and was not due to its oxidation-reduction potential or pH change. Leukemic cells were selectively affected at an L-ascorbic acid concentration attainable *in vivo* while normal hemopoietic cells were not suppressed.

INTRODUCTION

L-Ascorbic acid is not generally considered to be cytotoxic. Although there are reports concerning its toxicity in several tumor cell lines of animal origin (1, 10, 12), a direct cytotoxicity to human tumor cells has not been demonstrated. We here report that in a substantial proportion of patients with acute nonlymphocytic leukemia, the growth of leukemic cells in culture is suppressed by L-ascorbic acid.

It is generally known to be difficult to obtain *in vitro* growth of leukemic cell colonies with freshly aspirated bone marrow cells from patients with acute nonlymphocytic leukemia (15, 24). However, we have recently developed a culture method for the growth of human leukemic colonies (21). This is a modification of the agar culture method for normal myeloid colonies (commonly known as CFU-C) (2, 22) featuring daily feeding with new culture medium. The feeding technique was adopted from another culture method we developed previously for mouse myeloma (18) in which it was found to be important to feed the cultures daily with new culture medium containing L-ascorbic acid (19). In our recent study on acute nonlymphocytic leukemia (21), the daily feeding was also found to be very important for the growth of leukemic colonies in all the 8 patients studied. However, the addition of L-ascorbic acid to the feeding enhanced colony growth in only 2 of 8 patients. For the rest of the patients, although the feeding was needed, L-ascorbic acid did not enhance the colony growth (21).

We now have 28 patients with acute nonlymphocytic leukemia whose leukemic cells can be grown as colonies in the modified culture with feeding. Eight (28%) show a requirement for L-ascorbic acid in addition to the feeding. Among the rest, we have identified another subpopulation of 7 patients (25%) in whom the growth of leukemic colonies by the feeding is suppressed by the addition of L-ascorbic acid, and this phenomenon is the subject of our report.

MATERIALS AND METHODS

Patients. The clinical characteristics of the 7 leukemic patients in this study are shown in Table 1. Normal marrows used for controls were obtained from hematologically normal patients with solid tumors who were undergoing bone marrow aspiration as a part of staging workup. No patient had received prior treatment at the time of bone marrow aspiration for this study. Consent was obtained from all patients as designed and approved by the University of Kansas Human Subject Committee.

Culture Assay. The details of the culture method have been published (21). Briefly, the culture system consists of 2 layers of 0.3% agar in a 35-mm plastic Petri dish perforated at the bottom with 6 small holes. Bone marrow cells were placed in the upper of the 2 agar layers suspended in α medium free of L-ascorbic acid (Grand Island Biological Co., Grand Island, N. Y.) -containing 15% fetal calf serum and 15% leukocyte-conditioned medium prepared as described previously (21). Cultures were incubated at 37° with 7% CO_2 for 2 to 3 weeks. Throughout this period, each culture dish was taken out of incubator once daily to be fed from the top with 0.5 ml of L-ascorbic acid-free α medium containing 15% each of fetal calf serum and leukocyte-conditioned medium with or without freshly prepared L-ascorbic acid, GSH,[3] or HCl. In the previous study on the growth of mouse myeloma cells in culture, GSH was shown to enhance the effect of L-ascorbic acid (19), and both GSH and L-ascorbic acid were used in some experiments in this study. When the effect of L-ascorbic acid was under study, it had to be supplied to the cultures daily throughout the entire growth period because it has very short half-life in culture (4, 16). Likewise, GSH and HCl were also added daily when these were under study in order to make all the culture systems

[1] This investigation was supported by Grant R0-1 CA 20717 from NIH.

[2] Recipient of Research Career Development Award K04 CA 00534 from the National Cancer Institute. To whom requests for reprints should be addressed, at Division of Clinical Oncology, Department of Medicine, University of Kansas Medical Center, 39th and Rainbow Boulevard, Kansas City, Kans. 66103.

Received May 25, 1979; accepted January 4, 1980.

[3] The abbreviation used is: GSH, glutathione.

comparable. The fed medium passed through the agar layers and drained from the dish through holes at the bottom. Colonies were counted with an inverted microscope scoring only those with 50 or more cells.

It was shown in our previous study (21) that leukemic marrow cells gave rise to no or negligibly low numbers of colonies unless the cultures were fed daily and that the colonies which grew only with feeding were leukemic in origin as substantiated by the morphological, cytochemical, and chromosomal studies. On the other hand, normal bone marrow yielded the same number of myeloid colonies with or without feeding. To ensure that the colonies seen in the feeding culture were leukemic colonies and not normal myeloid colonies, simultaneous control cultures without feeding were performed on all 7 leukemic marrows. The number of colonies in these controls were only 5.2 ± 2% (S.E.) of the total number of colonies counted in the matched feeding culture. Therefore, even if all these colonies in the control cultures were normal myeloid colonies and all of these also grew in the feeding cultures, they represented a negligibly small fraction of the total number of colonies in the feeding culture and would not materially alter the results of the study. In addition, the colonies of 2 patients (Cases 1 and 2) were picked up and subjected to morphological and cytochemical studies as described (21). This directly confirmed leukemic origin of colonies of the patients in this study.

Table 1

Clinical data of 7 leukemic patients

Case	Age	Sex	Diagnosis	Blast cells[a] (%)	Chemotherapy	Complete remission[b]	Survival (mos.)
1	29	F	AML[c]	95	HU, AC[d]	No	1
2[e]	68	M	AML	80	None		9
3[f]	68	F	AML	30	None		5
4[g]	75	F	EL	20	None		4
5	74	M	AML	83	TG, VR, PR	No	1
6	48	F	AML	60	AD, VR, AC, PR	Yes	14+
7[h]	56	M	AMML	70	RZ, VR, AC, PR	No	8+

[a] Blast cell percentage of bone marrow aspirate used for culture study.
[b] Complete remission as defined by the disappearance of all evidence of disease with normal marrow (blast, <5%) and normal peripheral smear.
[c] AML, acute myelocytic leukemia; EL, erythroleukemia; AMML, acute myelomonocytic leukemia.
[d] HU, hydroxyurea; AC, 1-β-D-arabinofuranosylcytosine; None, no chemotherapy given; TG, 6-thioguanine; VR, vincristine; PR, prednisone; AD, adriamycin; RZ, Rubidazone.
[e] Abnormal karyotype (47 XYG+) identified in the bone marrow.
[f] She appeared to have oligoleukemia initially but developed a picture of full-blown acute leukemia 4 months later.
[g] The bone marrow showed marked dyserythropoiesis with megaloblastic change in addition to the blasts.
[h] The blast cells were once cleared from peripheral blood but not reduced below 5% in bone marrow.

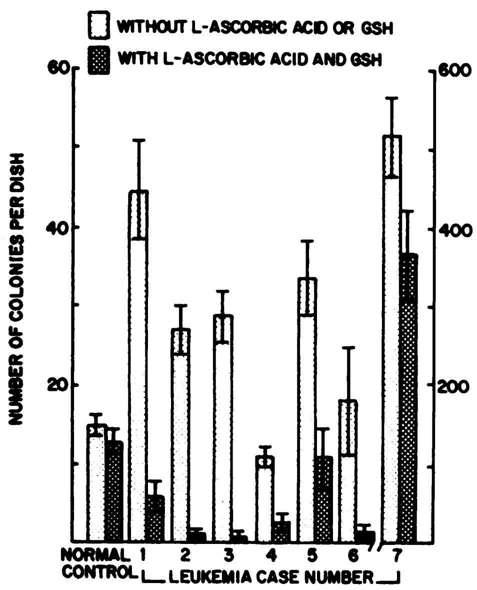

Chart 1. Number of colonies/dish cultured with or without both L-ascorbic acid (0.3 mM) and GSH (0.3 mM) in each of 7 cases of acute nonlymphocytic leukemia and in a hematologically normal patient. The normal control was cultured simultaneously with leukemia (Case 1). All cultures were plated with 5 × 10^5 nucleated cells/dish except Cases 1 and 2 in which 1 × 10^5 cells and 2 × 10^5 cells were plated, respectively. Case 7 had high plating efficiency requiring a separate *scale* as shown on the *right*. Student's *t* test for significant difference between the pairs of cultures showed $p < 0.01$ for Cases 1, 2, and 3; $0.01 < p < 0.05$ for Case 4; and $0.1 < p < 0.2$ for Cases 5, 6, and 7. In the latter 3 cases, however, the size of colonies was much smaller in the cultures with L-ascorbic acid and GSH (barely over 50 cells/colony) compared to that without L-ascorbic acid or GSH (over 100 cells/colony). Values are the mean of 5 dishes. *Bars*, S.E.

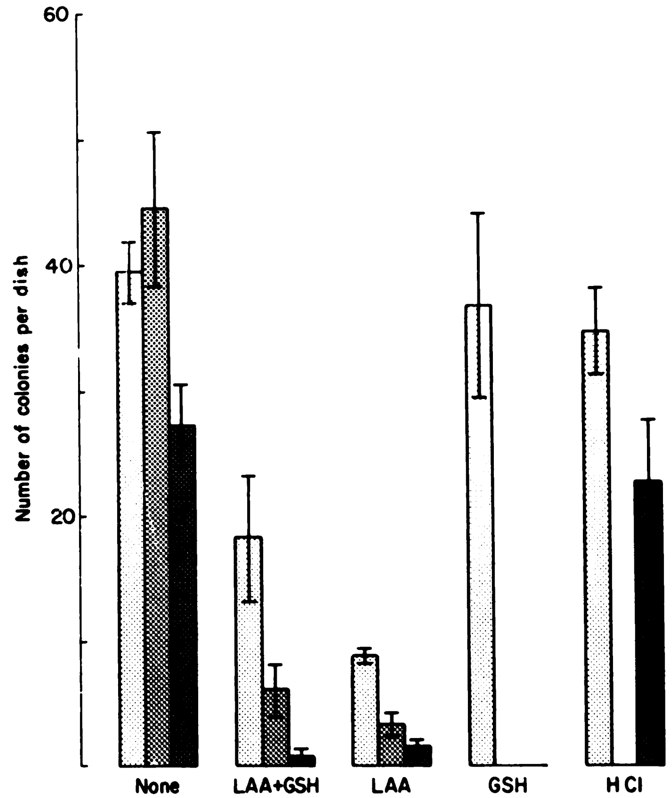

Chart 2. Number of colonies cultured with both L-ascorbic acid (*LAA*) and GSH, L-ascorbic acid alone, GSH alone, HCl alone, or no such addition (*None*) in 2 leukemic patients. The first 2 of each set of 3 columns (*lightly shaded*), 2 separate experiments on Case 1; and the last columns (*darkly shaded*), Case 2. L-Ascorbic acid and GSH were added at 0.3 mM each. HCl was added at 0.001 N. For the studies on GSH and HCl, only one and 2 experiments were performed, respectively. Values are the mean of 5 dishes. *Bars*, S.E.

RESULTS

The suppression of leukemic cell growth in the presence of L-ascorbic acid and GSH resulted in a reduction in the number of leukemic cell colonies to an average of 21 ± 9.2% (S.E.) of the controls as shown in Chart 1. This effect was reproduced in 2 cases tested. The colony suppression was consistently

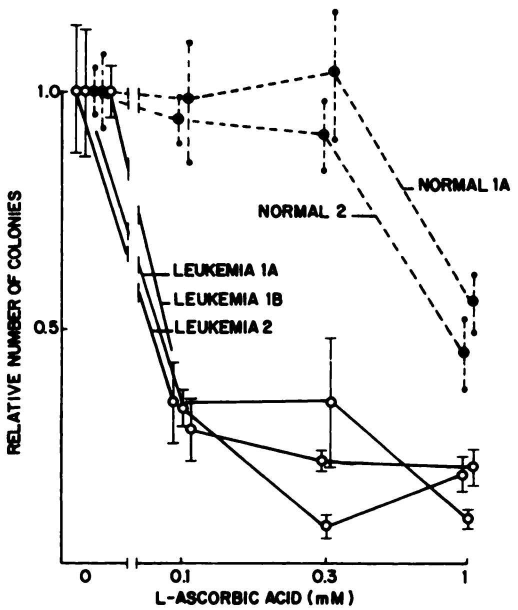

Chart 3. Relative number of colonies/dish in cultures with varying concentrations of L-ascorbic acid without GSH from 2 leukemic patients (Cases 1 and 2). The study was performed twice on Case 1 (*Leukemia 1A* and *Leukemia 1B*). Normal marrow was cultured simultaneously for both leukemic patients. The number of colonies are normalized to unity for the values obtained with no L-ascorbic acid. Absolute colony numbers were 44.7 and 39.5/1 × 10^5 cells for Leukemia 1A and 1B, respectively; 16/5 × 10^5 cells for Normal 1A; 57/2 × 10^5 cells for Leukemia 2; and 60.4/5 × 10^5 for Normal 2. *Points*, means of 5 dishes. *Bars*, S.E.

noted in 4 separate experiments in Case 1 and in 3 experiments in Case 2. Chart 2 shows that this growth suppression is due solely to L-ascorbic acid and not due to GSH which has an oxidation-reduction potential similar to that of L-ascorbic acid (14). Also, no growth suppression was noted when the medium was acidified with HCl to the pH of medium containing L-ascorbic acid. These data indicate that this growth suppression is a specific effect of L-ascorbic acid and not due to its oxidation-reduction potential or pH change. As shown in Chart 3, there is a clear separation between normal and leukemic cells over a wide range of L-ascorbic acid concentrations, indicating the selectivity for leukemic cells of this suppressing effect.

DISCUSSION

Our findings indicate that L-ascorbic acid suppresses the *in vitro* growth of leukemic cells in a substantial proportion (25%) of patients with acute nonlymphocytic leukemia. The clinical characteristics of these patients are shown in Table 1, but there do not appear to be any outstanding features which can distinguish this subpopulation of patients from the rest. The number of patients studied thus far may still be too few to detect any characteristic feature for these patients. One patient (Case 2) had an unequivocal picture of acute myelocytic leukemia with 80% blasts in bone marrow and an abnormal karyotype but had an unusually long survival without chemotherapy. However, a smoldering course in the acute leukemia with a high proportion of blasts in bone marrow has long been recognized (11). Another patient (Case 3) had low blast cell percentage and appeared to be oligoleukemic (9) at the time of initial culture study, but she developed a full-blown acute leukemia shortly. Also, it is known that the cell culture pattern of many with oligoleukemia is identical with that of typical acute leukemia (25), and the normal myeloid colony formation is not likely to occur unless the blast cell proportion is reduced below 20% (3). The blast cell proportion was low in another patient (Case 4), but she also had marked dyserythropoiesis, this being compatible with erythroleukemia (5). Response to the chemotherapy and survival are also difficult to assess. Chemotherapy was not given to 3 patients, and in one patient (Case 6), the chemotherapy was not intended for complete remission because of the number of clinical reasons such as advanced age, the possible smoldering course, etc.

It is most interesting to note that this suppressive effect on leukemic cells is specific to L-ascorbic acid. This suggests the possibility of a specific metabolic pathway being involved in this effect. There are possible mechanisms which could explain this effect. The lack of catalase may lead to cellular damage by the accumulation of H_2O_2 due to L-ascorbic acid (1, 10, 12). The lipid peroxide formed in mitochondria with the help of L-ascorbic acid (17, 26, 29) may cause injury to the lysosomal membrane with the release of enzymes (27) which in turn can lead to the damage of the cells (8). The increased level of cyclic adenosine 3′:5′-monophosphate induced by L-ascorbic acid (13) may inhibit cell growth (6, 13).

By far, the most important point in this study is that a profound suppression of leukemic cells can be achieved "without any damage to normal myeloid precursor cells" using the appropriate concentrations of L-ascorbic acid. This is because the major limiting factor in the drug treatment of cancer is the toxicity to normal tissue, especially to hemopoietic tissue. Among the 3 concentrations of L-ascorbic acid tested (Chart 3), the 2 lower ones (0.1 and 0.3 mM) are considered most appropriate covering the range of *in vivo* levels achievable with pharmacological doses in humans (28). Although the profound and selective suppression of leukemic cells was noted with these concentrations, we were tempted to test a higher concentration (1 mM) to see the maximum suppression of leukemic cells. No additional suppression of leukemic cells was noted. Instead, normal myeloid colonies started to be suppressed at this concentration which is, however, beyond the level achievable in humans, and therefore no clinical relevance can be given.

With this culture system (20) and another similar system (7, 23), it has been shown that there is a good correlation between *in vitro* cytotoxicity of chemotherapeutic drugs on malignant cells freshly obtained from the patients and the clinical response of the same patients to the same drugs. It is therefore conceivable that L-ascorbic acid might be selectively suppressive to the leukemic cells *in vivo* as *in vitro*. It is feasible to study this clinically because the lowest concentration shown to suppress leukemic growth *in vitro* (0.1 M) can be attained *in vivo* easily and safely with a pharmacological dose of L-ascorbic acid (28). If such a clinical study is contemplated, a preliminary *in vitro* test should be done in all patients to exclude those in whom L-ascorbic acid enhances the growth of leukemic cells.

ACKNOWLEDGMENTS

We thank Dr. B. Kimler for his valuable advice and L. Payne for her excellent technical assistance.

REFERENCES

1. Benade, L., Howard, T., and Burk, D. Synergistic killing of Ehrlich ascites carcinoma cells by ascorbate and 3-amino-1,2,4-triazole. Oncology (Basel), *23:* 33-43, 1969.
2. Bradley, T. R., and Metcalf, D. The growth of mouse bone marrow cells *in vitro*. Aust. J. Exp. Biol. Med. Sci., *44:* 287-299, 1966.
3. Bull, J. M., Duttera, M. J., Stashick, E. D., Northup, J., Henderson, E., and Carbone, P. P. Serial *in vitro* marrow culture in acute myelocytic leukemia. Blood, *42:* 679-686, 1973.
4. Feng, J., Melcher, A. H., Brunette, D. M., and Moe, H. K. Determination of L-ascorbic acid levels in culture medium: concentrations in commercial media and maintenance of levels under conditions of organ culture. In Vitro, *13:* 91-99, 1977.
5. French-American-British (FAB) Co-operative Group. Proposals for the classification of the acute leukemias. Br. J. Haematol., *33:* 451-458, 1976.
6. Gericke, D., and Chandra, P. Inhibition of tumor growth by nucleoside cyclic-3',5'-monophosphates. Hoppe-Seyler's Z. Physiol. Chem., *350:* 1469-1471, 1969.
7. Hamburger, A. W., and Salmon, S. E. Primary bioassay of human tumor stem cells. Science (Wash. D. C.), *197:* 461-463, 1977.
8. Hunter, F. E., Jr., Scott, A., Hoffsten, P. E., Gebicki, J. M., Weinstein, J., and Schneider, A. Studies on the mechanism of swelling, lysis, and disintegration of isolated liver mitochondria exposed to mixtures of oxidized and reduced glutathione. J. Biol. Chem., *239:* 614-621, 1964.
9. Jacquillat, C., Izrael, V., Weil, M., Chastang, C., Boiron, M., and Bernard, J. A study of 120 patients with oligo-blastic leukemia (OBL). Proc. Am. Assoc. Cancer Res., *16:* 103, 1975.
10. Josephy, P. D., Palcic, B., and Skarsgard, L. D. Ascorbate-enhanced cytotoxicity of misomidazole. Nature (Lond.), *271:* 370-372, 1978.
11. Knospe, W. H., and Gregory, S. A. Smoldering acute leukemia: clinical and cytogenetic studies in six patients. Arch. Intern. Med., *127:* 910-918, 1971.
12. Koch, C. J., and Biaglow, J. E. Toxicity, radiation sensitivity modification, and metabolic effects of dehydroascorbate and ascorbate in mammalian cells. J. Cell. Physiol., *94:* 299-306, 1978.
13. Lewis, S. Indirect biological influence of ascorbate on cyclic nucleotide-mediated hormonal activities. *In:* Vitamin C: Its Molecular Biology and Medical Potential, pp. 91-98. New York: Academic Press, Inc., 1976.
14. Loach, P. A. Oxidation-reduction potentials, absorbance bands and molar absorbance of compounds used in biochemical studies. *In:* G. D. Fasman (ed.), Handbook of Biochemistry and Molecular Biology, Ed. 3, Vol. 1, pp. 122-130. Cleveland: CRC Press, 1976.
15. Metcalf, D. Colony formation by myeloid leukemia cells. *In:* Hemopoietic Colonies, pp. 99-131. Berlin: Springer-Verlag, 1977.
16. Mohberg, J., and Johnson, M. J. Stability of vitamins in a chemically defined medium for 929-L fibroblasts. J. Natl. Cancer Inst., *31:* 603-610, 1963.
17. Ottolenghi, A. Interaction of ascorbic acid and mitochondrial lipids. Arch. Biochem. Biophys., *79:* 355-363, 1959.
18. Park, C. H., Bergsagel, D. E., and McCulloch, E. A. Mouse myeloma tumor stem cells: a primary cell culture assay. J. Natl. Cancer Inst., *46:* 411-422, 1971.
19. Park, C. H., Bergsagel, D. E., and McCulloch, E. A. Ascorbic acid: culture requirement for colony formation by mouse plasmacytoma cells. Science (Wash. D. C.), *174:* 720-722, 1971.
20. Park, C. H., Savin, M. A., Amare, M., and Hoogstraten, B. Chemotherapy sensitivity of human leukemic cells assessed by *in vitro* colony formation. Clin. Res., *25:* 345, 1977.
21. Park, C. H., Savin, M. A., Hoogstraten, B., Amare, M., and Hathaway, P. Improved growth of *in vitro* colonies in human acute leukemia using the feeding culture method. Cancer Res., *37:* 4595-4601, 1977.
22. Pluznik, D. H., and Sachs, L. The cloning of normal mast cells in tissue culture. J. Cell. Comp. Physiol., *66:* 319-324, 1965.
23. Salmon, S. E., Hamburger, A. W., Soehnlen, B., Durie, B. G. M., Alberts, D. S., and Moon, T. E. Quantitation of differential sensitivity of human tumor stem cells to anticancer drugs. N. Engl. J. Med., *298:* 1321-1327, 1978.
24. Spitzer, G., Verma, D. S., Dicke, K. A., and McCredie, K. B. Culture studies *in vitro* in human leukemia. Semin. Hematol., *15:* 352-378, 1978.
25. Spitzer, G., Verma, D. S., Dicke, K. A., Smith, T., and McCredie, K. B. Subgroups of oligoleukemia as identified by *in vitro* agar culture. Leukemia Res., *3:* 29-39, 1979.
26. Wills, E. D. Lipid peroxide formation in microsomes: general considerations. Biochem. J., *113:* 315-324, 1969.
27. Wills, E. D., and Wilkinson, A. E. Release of enzymes from lysosomes by irradiation and the relation of lipid peroxide formation to enzyme release. Biochem. J., *99:* 657-666, 1966.
28. Wilson, C. W. M. Clinical pharmacological aspects of ascorbic acid. Ann. N. Y. Acad. Sci., *258:* 355-376, 1975.
29. Wolfson, N., Wilbur, K. M., and Bernheim, F. Lipid peroxide formation in regenerating rat liver. Exp. Cell Res., *10:* 556-558, 1956.