Jpn. J. Cancer Res. 82, 1051–1055, September 1991

Cytotoxic Effects of Vitamin A in Combination with Vincristine, Daunorubicin and 6-Thioguanine upon Cells from Lymphoblastic Leukemic Patients

Rob Pieters,[1,3] Dieuwke R. Huismans,[1] Anne H. Loonen,[1] Karel Hählen[2] and Anjo J. P. Veerman[1]

[1]*Free University Hospital, Department of Pediatrics, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands and* [2]*Sophia's Children Hospital, Subdivision Haemato/Oncology, Erasmus University, Rotterdam, The Netherlands*

We studied whether isotretinoin potentiated the effects of vincristine (VCR), daunorubicin (DNR), and 6-thioguanine (6-TG) against cells obtained from 24 patients with acute lymphoblastic leukemia (ALL). Treatment with 5 μg/ml isotretinoin alone resulted in a leukemic cell survival of 82% ± 28.1%. So isotretinoin is toxic to ALL cells. Dose-response curves were obtained for VCR, DNR and 6-TG in the presence and absence of isotretinoin Isotretinoin showed additive leukemic cell kills in combination with VCR and DNR. When corrected for cell kill by isotretinoin alone, it appeared that isotretinoin did not significantly enhance leukemic cell kills by VCR, DNR and 6-TG. No differences were found between samples from patients at initial diagnosis and at relapse with respect to cell kill by isotretinoin alone and with respect to a possible synergistic effect of isotretinoin and the cytostatic drugs. It is concluded that isotretinoin has additive antileukemic effects in combination with VCR or DNR. However, isotretinoin does not potentiate the antileukemic effects of VCR, DNR and 6-TG against leukemic cells obtained from patients with ALL.

Key words: Vitamin A — Vincristine — Daunorubicin — 6-Thioguanine — Leukemia

Retinoids, analogs of vitamin A, have been shown to inhibit growth and to induce maturation in a large variety of tumor types. The results of many studies on this subject have been reviewed extensively by Lippman *et al.*[1,2] In these reviews it was suggested that retinoids have synergistic activity in combination with cytostatic drugs. The data to support this suggestion are, however, scarce and all such studies dealt with cell lines or animal models.[3–7] No studies using cells derived directly from patients have been reported as far as we know. We recently adapted the MTT assay to assess *in vitro* cytotoxic effects of chemotherapeutic drugs upon cells derived from leukemic patients.[8,9] With this assay, combinations of resistance-modifying agents with anticancer drugs were tested using cells from leukemic patients.[10] In the present study we evaluated whether vitamin A was capable of potentiating the cytotoxic effect of anticancer drugs against tumor cells obtained from patients with acute lymphoblastic leukemia (ALL).

MATERIALS AND METHODS

Reagents The vitamin A analog used was isotretinoin (Hoffman-La Roche, Basel, Switzerland). 6-Thioguanine (6-TG) was obtained from Sigma (St. Louis, USA); vincristine (VCR) and daunorubicin (DNR) from our hospital pharmacy; RPMI 1640 (Dutch modification), fetal calf serum (FCS), glutamine and antibiotics from Flow Laboratories (Irvine, Scotland); insulin, transferrin, selenite, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) from Sigma.

Methods Bone marrow and peripheral blood cells were obtained from 24 patients with ALL. Preparations of mononuclear cell suspensions and drug solutions were done as described earlier.[11] The MTT assay was performed as follows. Ninety-six-well microculture plates were filled with 40 μl of drug or isotretinoin or the combination of these two and stored at −20°C until use. Cryopreserved or freshly obtained leukemic cells were suspended in RPMI containing 20% FCS, 130 IU/ml penicillin, 130 μg/ml streptomycin, 0.16 μg/ml fungizone, 260 μg/ml gentamycin, 2.6 m*M* L-glutamine, 6.5 μg/ml insulin, 6.5 μg/ml transferrin, and 6.5 ng/ml sodium selenite. Cryopreservation does not alter the drug sensitivity results.[9,11] The wells contained 1.6×10^5 cells in a final volume of 100 μl. Plates were incubated in a humidified incubator in 5% CO_2 for 4 days at 37°C. After addition of 10 μl of MTT solution (5 mg/ml saline) and shaking for 1 min the plate was incubated for 6 h. MTT is reduced to a colored formazan by living but not by dead cells. Formazan crystals were dissolved with 100 μl of acidified (0.04 *N* HCl) isopropanol. The absorbance (*A*) of the wells was measured with a microplate reader (Titertek Multiskan MCC 340) at 540 nm. The *A* is linearly related to cell number.[8,10–12] Leukemic cell survival (LCS) was calculated by using the equation:

$$\text{LCS} = (A \text{ treated well/mean } A \text{ control wells}) \times 100\%.$$

[3] To whom correspondence should be addressed.

All experiments were performed in duplicate. Untreated control cells were run at 6 wells per test well. Cytostatic drugs were tested at the following final concentrations: VCR 0.05, 0.78 and 12.5 μg/ml; DNR 0.008, 0.125 and 2.0 μg/ml; 6-TG 1.95, 7.81 and 31.25 μg/ml. The concentration of isotretinoin was derived from dose-response curves of a very wide range of concentrations of isotretinoin tested in 5 ALL samples (Fig. 1). A concentration of 5 μg/ml was chosen to be used in further studies because this concentration was not so high as to kill all ALL cells, which would make further combining experiments with cytostatic drugs useless. On the other hand, we did not want to use excessively low concentrations, which could have resulted in the possibility of missing synergistic effects.

The synergistic effects have been calculated as described by Weisenthal *et al.*[13] The effect was defined as a synergistic effect if the cell kill by the combination of isotretinoin and a drug (e.g. DNR) was more than the product of the cell kill by isotretinoin and DNR tested separately. For instance, if isotretinoin results in 80% cell survival and DNR in 50% cell survival, the combination of isotretinoin and DNR must result in a cell survival $<40\%$ to be defined as synergistic. The correction formula for the leukemic cell survival (LCS) is as follows:

$$\text{Corrected LCS} = \frac{(\text{LCS with retin} + \text{DNR}) \times 100\%}{(\text{LCS with retin})}$$

For instance, if isotretinoin results in 80% cell survival, DNR in 50% cell survival, and the combination of isotretinoin and DNR results in 40% cell survival, then the corrected LCS is 50%. This means that in this example there is only an additional effect and no synergistic effect, because the cell kill by DNR alone was also 50%.

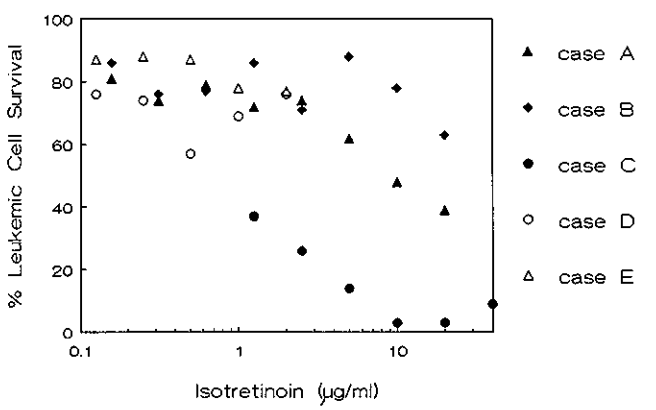

Fig. 1. Dose-response curves of isotretinoin upon ALL cells obtained from 5 children with ALL.

The results could be evaluated in 17 cases, 10 samples from untreated ALL patients at initial diagnosis and 7 from patients at relapse. In 7 cases the control cell survival was too low, i.e., A of the control cells was <0.050, to evaluate the results.[11]

Statistics The Wilcoxon matched-pairs signed-ranks test and the Mann-Whitney U test (MWU) were used for two-tailed testing at a level of significance of 0.05.

RESULTS

Cells from ALL patients showed a wide interindividual range of sensitivity to isotretinoin alone (Fig. 2). Treatment with 5 μg/ml isotretinoin resulted in an LCS of $82\% \pm 28.1\%$ (mean $\pm$ SD; $n=17$). The effect of isotretinoin alone on the LCS was not significantly different between relapsed ($85\% \pm 10.6\%$) and initial ALL samples ($80\% \pm 35.4\%$). Sixteen out of the 17 LCS values were in the range of 35–109%. There was one out-of-range value of 160%. Leukemic cells from this ALL patient were characterized by colony formation after 4 days of culture.

Dose-response curves were obtained for VCR, DNR and 6-TG in the presence and absence of isotretinoin (Figs. 3–5). Isotretinoin had significant additive cyto-

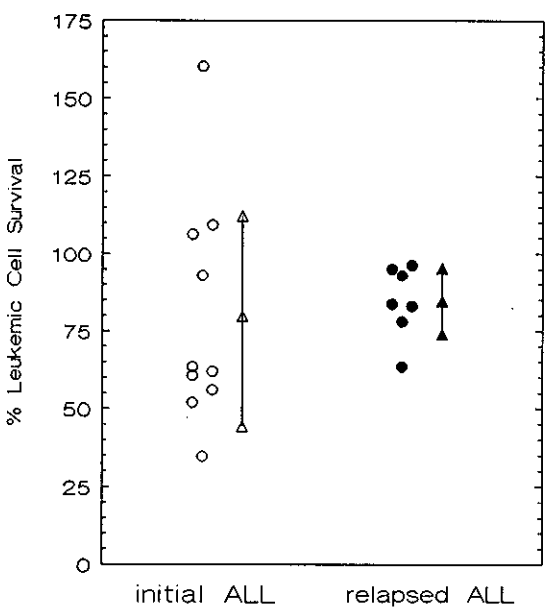

Fig. 2. Effects of 5 μg/ml isotretinoin alone upon leukemic cell survival of 10 samples from patients with untreated ALL at initial diagnosis and 7 samples from patients with relapsed ALL. Circles represent individual values and triangles represent mean $\pm$ SD.

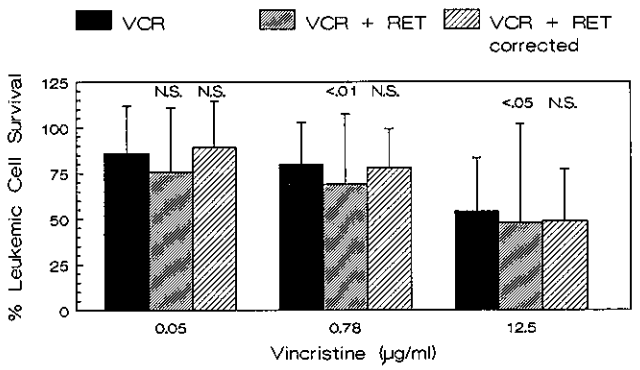

Fig. 3. Effects of vincristine (VCR) upon leukemic cell survival (LCS) in the presence and absence of 5 μg/ml isotretinoin (RET). Values given are mean $\pm$ SD of samples from patients with ALL. The three bars at each concentration represent LCS values after incubation with e.g. VCR alone (VCR), or after incubation with VCR plus isotretinoin, not corrected (VCR + RET) or corrected for the cell kill by isotretinoin alone (VCR + RET corrected). The LCS of the combination of drugs is compared with that of VCR alone: the *P*-values are shown in the figure; N.S. = not significant.

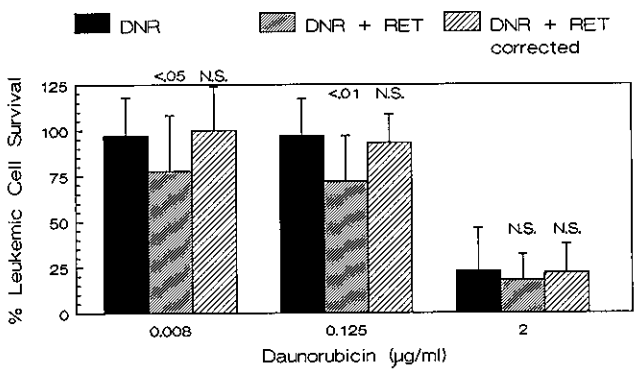

Fig. 4. Effects of daunorubicin (DNR) upon leukemic cell survival in the presence and absence of 5 μg/ml isotretinoin. Symbols as in Fig. 3.

toxic effects in combination with VCR at 0.78 μg/ml ($P=0.01$) and 12.5 μg/ml ($P<0.05$) and with DNR at 0.008 μg/ml ($P=0.05$) and 0.125 μg/ml ($P<0.01$). Synergistic effects were not found: when corrected for the cell kill by isotretinoin alone, isotretinoin did not significantly enhance leukemic cell kill by VCR, DNR, or 6-TG. At none of the 3 concentrations of the 3 drugs were significant differences be-tween initial samples and relapsed samples found with respect to a possible synergistic effect of isotretinoin and cytostatic drug.

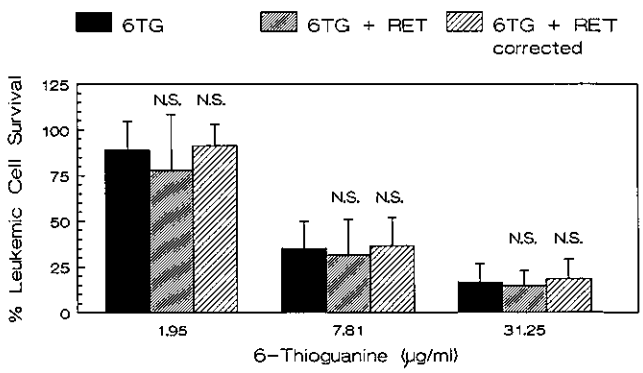

Fig. 5. Effects of 6-thioguanine (6-TG) upon leukemic cell survival in the presence and absence of 5 μg/ml isotretinoin. Symbols as in Fig. 3.

DISCUSSION

Several studies have provided evidence that analogs of vitamin A can enhance tumor cell kill by anticancer drugs. Two studies showed that retinoids enhanced the cytocidal activities of vinca alkaloids on melanoma and leukemia cell lines, most probably by increased accumulation of the alkaloids.[5,7] In the *in vivo* study with mice by Nakagawa *et al.*[4] retinol palmitate enhanced the antitumor effects of 5-fluorouracil (5-FU), methotrexate (MTX), and ACNU but not of adriamycin (ADM) or 6-mercaptopurine (6-MP) against sarcoma 180. With P388 leukemia an enhanced effect was seen for 6-MP, MTX, ADM, ACNU, and cisplatinum, but not for 5-FU. In an earlier study by the same group of investigators, the *in vitro* antitumor effect of 6-MP was enhanced by retinoids against HeLa cells *in vitro* and against L1210 leukemia *in vivo*.[3] In the report of Bregman *et al.*[6] the combination of dexamethasone and retinoic acid effected a synergistic inhibition of colony formation of a murine but not a human melanoma cell line.

However, in all five studies mentioned above cell lines or animal models were used. To our knowledge the current paper is the first dealing with the potentiating effect of vitamin A upon cell kill by cytostatic drugs using cells directly obtained from patients. Significant additive cell kill was found for the combination of isotretinoin with VCR or DNR. In the case of 6-TG the combination did not lead to a significant additive effect but the number of samples tested was only 9. When the LCS values were corrected for the cytotoxic effect of isotretinoin alone, no synergistic cell kill by isotretinoin and VCR, DNR, or 6-TG could be detected. Theoretically, differences between initial and relapsed samples could obscure such a synergistic effect in one or both groups. However, no

differences were found between relapsed and initial samples. So the results are unequivocal: isotretinoin showed additive effects in combination with DNR or VCR but did not potentiate the antileukemic effects of VCR, DNR and 6-TG.

Our results differ from the findings in the three reports of the Japanese investigators[3-5] described above. This might be due to several factors. First and probably very important, those authors used tumor cells from *in vitro* and *in vivo* models which differ from leukemic cells from patients. ALL cells derived from patients have a very low clonogenic capacity *in vitro,* making clonogenic assays impossible to use for *in vitro* chemosensitivity studies. The use of different kinds of assays in cells from leukemic patients has been reviewed recently by several authors.[12, 14-16] Because of its favorable clinical correlations and practical advantages, short-term assays such as the MTT assay appear to be at least as valuable in the clinical situation as long-term clonogenic assays. This is especially true for lymphatic leukemias, because these cells can not be tested in clonogenic assays. Recently we showed that drug resistance at initial diagnosis, determined with the MTT assay, is of prognostic relevance in childhood ALL.[17] The fact that the results of the present study show some discrepancies with those of other authors might be explained by the fact that we used ALL cells from patients with a low clonogenic capacity while others only used experimental cell lines with a high clonogenic capacity.

In the second place, the Japanese researchers found that the potentiating effect of vitamin A was higher in VCR-resistant sublines than in VCR-sensitive lines. In the present study no differences were detected between cells from initial and from relapsed patients with respect to vitamin A effects. The drug-resistance profiles of the relapsed patients used in this study are described elsewhere.[18] Cells from the relapsed ALL patients were significantly more resistant to 6-TG and DNR, but not to VCR, compared to cells from ALL patients at initial diagnosis. None of these patients showed the multidrug resistance phenotype, which is characterized by a decreased accumulation of VCR and DNR. If vitamin A would act by influencing this accumulation, as suggested by two independent groups,[5, 7] then this could partly explain the discrepancies with the results of our current study. On the other hand, the other authors reported synergistic effects in the sensitive cell lines and also for drugs not involved in the multidrug resistance phenotype, such as antimetabolites.

In one specimen, isotretinoin increased leukemic cell survival. Leukemic cells from this ALL patient were characterized by colony formation after 4 days of culture, which is very unusual in culturing cells from ALL patients. These cells also showed an increased survival upon *in vitro* treatment with VCR and prednisolone.[11] Retinoids have been observed to stimulate growth in some tumor cell lines too.[1] The explanation for this phenomenon is still unclear.

We conclude from our study that isotretinoin shows additive antileukemic effects in combination with DNR or VCR. However, significant synergistic effects were not found, implying that isotretinoin does not potentiate the antileukemic effects of VCR, DNR and 6-TG against leukemic cells obtained from patients with ALL.

ACKNOWLEDGMENTS

This research was supported by the Dutch Cancer Society (IKA 87–17).

(Received April 1, 1991/Accepted June 22, 1991)

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