Perturbation of Generation Cycle of Human Leukemic Blast Cells by Cytostatic Therapy In Vivo: Effect of Corticosteroids

By PETER ERNST and SVEN-AAGE KILLMANN

The in vivo cell cycle effects of intensive short-term corticosteroid treatment on bone marrow lymphoblasts have been studied in five patients with acute lymphoblastic leukemia. In repeated bone marrow samples, the following parameters were determined: mitotic index, stathmokinetic index after vincristine, $^3$H-thymidine labeling index and single cell DNA content. It is concluded that prednisone has a general destructive effect both on actively proliferating and on quiescent leukemic lymphoblasts. Besides, prednisone has an additional effect on proliferating lymphoblasts which manifests itself by a decreased influx from $G_1$ phase into DNA synthesis phase. From the present data it cannot be decided whether this is due to a depopulation of the $G_1$ pool or results from an arrest of cells in $G_1$. No evidence has been found for selective cell death during DNA synthesis. Some implications of this cytokinetic perturbation for therapeutic combination of corticosteroids with methotrexate and vincristine are discussed.

RECENT STUDIES IN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) indicate that adrenal corticosteroids exert profound effects on the cell cycle of lymphoblasts; notably, the fraction of lymphoblasts in DNA-synthesis becomes markedly reduced even after short term therapy.$^{1,2}$ In this paper, additional observations on the cytokinetic effects of corticosteroids on leukemic lymphoblasts are presented.
Table 1.—Clinical and Therapeutical Data on Patients Studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age and Sex</th>
<th>Previous Treatment</th>
<th>Treatment During Study</th>
<th>Blood Blast Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>OBJ</td>
<td>21 M</td>
<td>Nil</td>
<td>200 mg. P, then 25 mg. P × 3 with 6 hr. intervals. VCR 5.5 mg. 16 hrs. after start of P</td>
<td>— —</td>
</tr>
<tr>
<td>OBJs</td>
<td>— —</td>
<td>6-MP 100 mg. daily, D/C 2 days before study</td>
<td>200 mg. P, then 25 mg. P × 3 with 6 hr. intervals</td>
<td>— —</td>
</tr>
<tr>
<td>KII</td>
<td>27 F</td>
<td>Nil</td>
<td>50 mg. P, then 15 mg. P × 3 with 6 hr. intervals</td>
<td>64,000 26,500</td>
</tr>
<tr>
<td>HPS</td>
<td>61 M</td>
<td>10 mg. P daily until study. MTX 50 mg. weekly, D/C 3 weeks before study</td>
<td>150 mg. P, then 25 mg. P × 3 with 6 hr. intervals</td>
<td>10,500 14,000</td>
</tr>
<tr>
<td>BJ</td>
<td>17 M</td>
<td>Nil</td>
<td>200 mg. P, then 25 mg. P × 4 with 6 hr. intervals</td>
<td>9,000 3,000</td>
</tr>
<tr>
<td>KFL</td>
<td>15 M</td>
<td>Nil</td>
<td>140 mg. M-PL, then 20 mg. M-PL × 3 with 6 hr. intervals</td>
<td>79,000 14,000</td>
</tr>
</tbody>
</table>

6-MP, 6-mercaptopurine; MTX, methotrexate; VCR, vincristine; P, Prednisone; M-PL, methylprednisolone.

Materials and Methods

Five patients with ALL were studied; one patient was studied twice. The patients are listed in Table 1, together with some pertinent data. Four patients were given 50–200 mg. of prednisone (P) orally followed every 6 hours by 15–25 mg. P during a period of 18 hours. In one case, 140 mg. of methylprednisolone (M-PL) was given intravenously, followed by 20 mg. at 6 hour intervals for 18 hours. Serial bone marrow samples were obtained during the treatment period; smears were prepared without delay and fixed in absolute methanol for 15 minutes and stained with May-Grünwald-Giemsa. The remainder of the aspirate (1–2 ml.) was incubated with 2 μCi. of 3H-thymidine (3H-TDR) (specific activity, 1.9 Ci./mM, New England Nuclear Corp.) for 1 hour at 37°C, using 0.5 ml. of 1 per cent EDTA in 0.7 per cent saline as an anticoagulant. After this, smears were prepared, fixed as described above, and processed as previously described for autoradiography with Kodak NTB-2 dipping emulsion. Exposure time was 1 week. The percentage of 3H-TDR labeled lymphoblasts (LI) was determined by counting at least 1000 cells. The percentage of blast cell mitoses (late prophase to late telophase) (MI) was determined by counting at least 3000 blast cells in the direct bone marrow smears.

In some samples autoradiography was combined with ultramicrospectrophotometric determination of single cell DNA content using the Zeiss UMSP-I ultramicrospectrophotometer. Cells were cytologically classified in Giemsa stained autoradiographs and recorded on a photomap. Next, the Giemsa stain was removed with 5 per cent cold trichloroacetic acid for 1 minute followed by absolute methanol until no stain was left. The silver grains were then removed. Following this, the cells were Feulgen stained (hydrolysis with 5 N hydrochloric acid at 25°C for 25 minutes followed by freshly prepared Schiff’s reagent at pH 2.5 for 2 hours). Finally, the slides were rinsed 3 times (10 minutes each time) with freshly prepared SO2 water. Feulgen absorption was measured at maximal absorption, usually at 560 nm. For segmented granulocytes and leukemic blast cells unlabeled with 3H-TDR, the coefficient of variation of Feulgen absorbance is 3 per cent. This procedure was used when the primary purpose was to measure the DNA content of 3H-TDR labeled cells.
PERTURBATION OF CYCLE OF LEUKEMIC BLAST CELLS

Table 2.—Data on 3H-TDR Labeling Index (LI) and Mitotic Index (MI) of Leukemic Bone Marrow Lymphoblasts During Corticosteroid Treatment*

<table>
<thead>
<tr>
<th>Name</th>
<th>Hours After Corticosteroid</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3½</td>
</tr>
<tr>
<td>OBJ₁</td>
<td>LI 27.6</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>MI 1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>OBJ₂</td>
<td>LI 27.0</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td>MI 1.18</td>
<td>1.20</td>
</tr>
<tr>
<td>KII</td>
<td>LI 6.1</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>MI 0.37</td>
<td>0.33</td>
</tr>
<tr>
<td>HPS</td>
<td>LI 25.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MI 1.35</td>
<td></td>
</tr>
<tr>
<td>BJ</td>
<td>LI 9.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>MI 0.57</td>
<td>0.57</td>
</tr>
<tr>
<td>KFL</td>
<td>LI 15.0</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>MI 0.80</td>
<td>0.93</td>
</tr>
</tbody>
</table>

* Abbreviations as in Table 1.

In cases where the aim was to measure DNA in unlabeled cells, the method of Cooper et al. was used.

RESULTS

The results are presented in Table 2. In one patient (HPS) who had been on long term P treatment and at the time of the study proved to be clinically resistant to higher doses of this drug, the LI was not affected by therapy. In the remaining cases, all of which responded clinically to P therapy, the LI showed a substantial decrease within the first 24 hours of P administration. From cases OBJ₁ and KFL (Table 2 and Fig. 1) it appears that there is a certain lag period (more than 3.5–4.5 hours but less than 8 hours) before the decline in LI sets in. It should be noted that the MI falls considerably later (Table 2). MI equal to the pretreatment MI was observed as late as 21 hours after start of therapy (case B.J.). In one case (OBJ₁, Fig. 2), VCR was given 16 hours after start of P; this was followed by a marked rise in MI.

Fig. 1.—Effect of prednisone on 3H-TDR labeling index of leukemic lymphoblasts. (A) at time of diagnosis, (B) in first relapse. In (A) vincristine was given 16 hours after start of prednisone.
Fig. 2.—Patient OBJa. 

$^3$H-TDR labeling index ($L_i$) and mitotic index ($M_i$) of leukemic lymphoblasts. Prednisone was started at $T = 0$. The labeling index falls markedly; in contrast, the mitotic index remains unchanged, and vincristine (VCR) given at 16 hours produces a rapid and substantial increase in mitotic figures.

Figures 3 and 4 show the nuclear DNA content of $^3$H-TDR labeled cells before and 16 hours after P. It is seen that after P there is a relative lack of cells with a DNA content corresponding to the early part of the DNA-synthesis phase. Before therapy, the ratio between the cells with a 14–22 arbitrary unit DNA content and those with a 23–32 DNA content was 104:86. After 16 hours of P, this ratio changes significantly ($p < 0.01$) to 27:66.

Fig. 3.—Distribution of DNA content in $^3$H-TDR-labeled cells before prednisone. AU, arbitrary units.
DISCUSSION

At the time of diagnosis, the majority of leukemic blast cells are in a quiescent nonproliferating state. The vigorous decrease in leukemic cell population size because of intensive short term corticosteroid therapy, illustrated here by the fall in blood blast concentration (Table 1) and the overnight disappearance of a huge mediastinal mass in one of the patients (OBJa) shows that the effect of P cannot be restricted to the actively proliferating blast cells; in that case, the response would be less rapid. It is thus clear that P destroys quiescent lymphoblasts which is also in accordance with the reported direct killing of lymphatic cells by adrenocorticosteroids. This then raises the question of whether the destructive effect of P is limited to the quiescent lymphoblasts. If this were the case, the proportion of actively proliferating lymphoblasts would rise during P treatment, which would result in increases of LI and MI. This was not observed. The constancy of the MI for some time after start of treatment suggests that the proliferating leukemic cells were affected to the same degree as the quiescent cells. However, superimposed on this, P appears to have an additional effect on the proliferating cells. The evidence for this is the gradual decline in LI and the (delayed) decrease in MI. The mechanism of this appears to be that P reduces the efflux from G1 into S, as also suggested by Lampkin et al.2

Evidence for an arrested influx into S comes from the following observations:

1. The decrease in LI is gradual. This would be expected if there was little or no entry into S but normal progression through and exit from S. In that case, very low LI-values would be expected only after a period of time corresponding to the duration of S. In the present study, such low values were observed after about 21–24 hours. The duration of S in leukemic blast cells is
about 15–20 hours in most studies. The few hours' lag period before the LI begins to fall may reflect the time needed for P to be absorbed, enter the cells and exert its biochemical action. It has been reported that P may interfere with cellular uptake of nucleosides. We have previously demonstrated, however, that, under the conditions of the present studies, the fraction of 3H-TDR-labeled cells corresponds to the number of cells in S phase as determined by ultramicrospectrophotometric measurements of cellular DNA content.

2. The decrease in MI lags considerably behind the drop in LI. This suggests that there is no preferential killing of proliferating cells while they are in S and that cells which are in S when P treatment is started complete their DNA synthesis and enter mitosis. This is further supported by the data of Fig. 2. Here, VCR was given 16 hours after start of P at a time when the LI had dropped from an initial value of 27.6 to 5.8; nevertheless, VCR resulted in a rapid and marked accumulation of mitotic figures; the increment in arrested metaphases is identical with the increment in other studies where only VCR has been administered. This proves a substantial and probably undisturbed efflux from G2 and S during this phase of P treatment.

3. Measurements on the position of labeled cells in S phase (Figs. 3 and 4) show that before P there are slightly more cells in the first than in the second half of S phase (104 vs. 86 cells); 16 hours after P, the position of labeled cells in S is shifted towards the end of DNA synthesis. Sixty-six of 93 cells are located in the second half of S. Again, this is consistent with a reduced entry into S (relative depopulation of early S phase) and a normal progression through DNA synthesis of cells which were in S when treatment was started.

In conclusion, apart from its more general cytotoxic effect on proliferating and quiescent lymphoblasts, P severely curtails the entry from G1 to S in proliferating lymphoblasts. From the present data it cannot be decided whether this is due to a selective killing of cells in G1, or whether P blocks the transition from G1 to S. From observations in other cell systems, it appears likely that the action of P is to inhibit the progression of cells from G1 to S. Thus, hydrocortisone has been demonstrated to prolong the late part of G1 in Hela Chessen cells; this effect is reversible. In squamous epithelial cells of the mouse forestomach, a similar arrest in G1 is produced by hydrocortisone, as well as by actinomycin D; hence, the mechanism of action of corticosteroids may be an inhibition of RNA synthesis. This is supported by evidence which shows that hydrocortisone inhibits the formation of RNA polymerase although final evaluation of this point will depend on biochemical studies on synchronized cells. One may speculate that the ultimate effect of corticosteroids is to inhibit synthesis of DNA polymerase. However, it should not be overlooked that the determining biochemical effect may vary from one cell system to another. Thus, besides G1 arrest, inhibition of DNA synthesis has been reported in murine lymphatic leukemic cells. It is of interest to note that the cell cycle effects of corticosteroids are not only observed in lymphoid cells but also in epithelial cells, at least in animals and in cultures derived from a human epithelial carcinoma (HeLa). In this context, the gastric ulcers produced by steroids come to mind. However, other systems such as neutrophil precursors are apparently not influenced. This is important from a therapeutic point of view.
PERTURBATION OF CYCLE OF LEUKEMIC BLAST CELLS

It is probable that studies of the effect of cytostatic and cytocidal drugs on the cell cycle of neoplastic cells will be helpful in designing better chemotherapeutic management of malignant growth. From the present data, some suggestions concerning the use of drug combination in ALL may be made, although it is clear that additional studies are needed, and that therapeutic extrapolations from cytokinetic observations must ultimately be checked by controlled clinical trials.

Methotrexate (MTX) and P

Administration of MTX intravenously at the same time as or 3–4 hours after an initial large dose of P seems rational. P will reduce the influx of cells into S; on the other hand, MTX, as judged from studies in myeloblastic leukemia does not interfere with entry into S but arrests the cells in S. If MTX is given later than a few hours after high dose P treatment has been initiated, less antileukemic effect of MTX is predicted since, at this time, the fraction of lymphoblasts in S has decreased if the cells are sensitive to P; hence the target population for MTX is reduced. Moreover, cells that might overcome the G1 block of P and later enter S will probably not be affected by MTX given while they were in G1. Myeloblasts which are in G1 at the time of MTX injection appear to progress through S without apparent difficulty. Consequently, if MTX is given some time after initiation of P, one may expect reduced MTX effect on the leukemic lymphoblasts but full effect (i.e., toxicity) on other tissues. If P and MTX are to be combined in a course of therapy, it may be considered to pause with corticosteroid for some time prior to the injection of MTX. However, this remains to be studied. It should be noted that the LI of lymphoblasts remains low during continued corticosteroid therapy, and that buildup of the S-pool after cessation of P would appear to be slow (Tables 1 and 2).

Vincristine (VCR) and P.

The stathmokinetic action of VCR sets in rapidly; the duration of its action is not yet well defined. In leukemic blast cells, many arrested metaphases are still observed 24 hours after VCR however, at this time or somewhat before, telophases reappear. This indicates that the effect on cells entering mitosis lasts for 24 hours or somewhat less. The drug appears to affect mitosis in cells which at the time of VCR injection are in G2, S and, perhaps, late G1. It therefore appears rational to use VCR at the time P is started. VCR will arrest in mitosis cells which were in S or G2 at start of treatment and P will prevent the replenishment of the S-pool from G1. If VCR is given later, the S-pool will be more or less depleted because of the action of P, and therefore total leukemic cell arrest in mitosis can be expected from VCR. After intensive P therapy for about 24 hours, the therapeutic gain by a dose of VCR might be quite limited if the main mechanism of action of VCR is mitotic arrest, as is generally believed. Whether it might be advantageous to administer VCR before P will depend on several factors and needs to be studied. This
also applies to the use of intermittent VCR during continuous P medication. Conceivably, increased effect might be obtained by withholding P for a limited time prior to VCR.

REFERENCES


15. Ernst, P., and Killmann, S.-Aa.: To be published.


Perturbation of Generation Cycle of Human Leukemic Blast Cells by Cytostatic Therapy In Vivo: Effect of Corticosteroids

PETER ERNST and SVEN-AAGE KILLMANN