Human Leukocyte Metabolism in Vitro

II. The Effect of 6-Mercaptopurine on Formate-$C^{14}$ Incorporation into the Nucleic Acids of Acute Leukemic Leukocytes

By JAMES L. Scott, JOSEPH V. MARINO AND E. PETER GABOR

The mechanism of action of 6-mercaptopurine (6-MP) has been studied extensively, particularly in bacteria and mouse-propagated neoplastic cells of various strains in which the biochemical effect of the analog can be correlated with inhibition of cell growth. Although the results in various cell systems have been somewhat different, a majority of these investigations suggest that 6-MP inhibits one or more of the reactions required for synthesis of the purine ring or for the interconversion of the physiological purines.

A previous study from this laboratory indicated that in vitro formate-$C^{14}$ incorporation into the nucleic acids of human leukocytes reflected de novo purine synthesis by these cells, and was also useful as a measure of DNA thymine synthesis. The present study was undertaken to explore the effect of 6-MP on these metabolic processes in human acute leukemic leukocytes and to correlate the results with the clinical effects of the analog.

Materials and Methods

Clinical Material

Because of the number of cells required for analysis, the study was limited to leukemic patients with elevated blood leucocyte counts. The criteria of Boggs, Wintrobe and Cartwright were used in classifying the leukemias morphologically. An adequate trial of therapy with 6-MP was considered to consist of the administration of 2.5 mg./Kg. of body weight per day for 4 weeks or more, under conditions allowing a thorough clinical and laboratory evaluation of the drug’s effect. The criteria for remission were those of Frei and co-workers. Complete resistance to 6-MP was judged present if the number of circulating blasts increased or remained unchanged during therapy and there was other evidence of progressive disease.

Methods

Venous blood was drawn into a heparinized syringe, mixed with 0.5 volume of 6 per cent dextran in 0.9 per cent saline, and allowed to stand until the red cells had sedimented.
The leukocytes were concentrated by low-speed centrifugation of the supernatant plasma-dextran layer and resuspended in a pH 7.4 medium composed of 50 per cent pooled human serum and Hanks' solution, in a concentration of approximately 30,000 leukocytes per mm. For the duplicate control and 6-MP-treated incubations, 40 ml. of this suspension were divided between four flasks containing 10 μc. of sodium formate-C\(^{14}\). Chromatographically-pure 6-mercaptopurine monohydrate (Mann Research Laboratories) added to two flasks to a final concentration of \(6 \times 10^{-4}\) mM/ml.; the choice of this concentration was based on the results of Davidson's study of L-1210 mouse leukemia cells.\(^7\)

After incubation for 6 hours in a metabolic shaker at 37 C. in open flasks, the cells were washed three times in 0.9 per cent saline and homogenized in cold 0.5 M perchloric acid to extract the acid-soluble nucleotides and precipitate the cell proteins and nucleic acids. From this precipitate, the nucleic acids were extracted into 10 per cent sodium chloride, maintained at neutral pH, in a boiling water bath. The dissolved nucleic acids were reprecipitated from the salt solution by the addition of 8 volumes of cold ethanol, and the precipitate was dried with alcohol and ether. The nucleic acids were dissolved in 0.1 N KOH and RNA hydrolyzed to soluble nucleotides by incubation at 37 C. for 24 hours. After chilling, DNA was precipitated from the solution by neutralization with HCl. The RNA nucleotides were recovered from the neutral supernatant after conversion to their barium salts by precipitation in cold ethanol. The DNA and RNA nucleotides were further hydrolyzed to their free bases in concentrated perchloric acid at 100 C., and the bases were separated by two-dimensional paper chromatography in isopropanol-HCl-water and butanol-ammonia. After location of the bases under ultraviolet illumination, the radioactivity in paper segments containing the bases was determined in a thin-window gas-flow counter (Nuclear-Chicago) with an overall counting efficiency of 8.4 per cent. Sufficient counts were recorded to reduce the statistical error of counting to less than 3 per cent; the background radioactivity of each chromatogram was similarly determined and subtracted from the counting rates of the samples. The counted samples were eluted into 0.1 N HCl; the base content of the eluates were estimated using ultraviolet spectrophotometry, and the specific activities of the samples were calculated.\(^4\)

**Presentation of Data**

The experiments were done over a period of several years, using supplies of radioactive formate ranging in specific activity from 1.5 to 9.5 μc./μM with resulting formate-C\(^{14}\) concentrations of 6.6 to 1.1 μM/10 ml. incubation. No relationship between these concentrations and the extent of formate incorporation into the cells was evident but, for uniformity, the results are presented as the relative specific activity of the individual bases (RSA), calculated from the formula

\[
\frac{\text{cpm/μM of base}}{\text{cpm/μM of added formate-C}^{14}} \times 100
\]

The specific activity of the DNA purines was in every case parallel to, and less than, 10 per cent of the specific activity of the RNA purines. Because this minor amount of incorporation could result from the presence of contaminating RNA purines,\(^4\) the data pertaining to the DNA purines have been omitted to simplify presentation.

The statistical significance of differences between the specific activities of the individual nucleic acid bases of the control and treated cells was tested after conversion of the numbers, as cpm/μM, to their logarithms. This normalizes the exponential relationship of counting rates and differences between repeated counts of the same sample. The variance (Sd) of differences between the results of duplicate incubations was estimated by the formula

\[
\text{Sd} = \sqrt{\frac{\frac{\Sigma d^2}{n} - \left(\frac{\Sigma d}{n}\right)^2}{n-1}}
\]
Fig. 1.—Influence of 6-mercaptopurine on formate-C\(^{14}\) incorporation into leukemic leukocyte RNA purines and DNA thymine. Solid bars, RNA adenine; open bars, RNA guanine; hatched bars, DNA thymine. Data points indicate the range between duplicate 6-hour incubations; C, control incubations; M, 6-MP-treated incubations. In AML experiments, formate-C\(^{14}\) specific activity 1.79 mc./mM, concentration 5.6 \(\mu\)M/10 ml.; in ALL experiments, formate-C\(^{14}\) specific activity 9.5 mc./mM, concentration 1.1 \(\mu\)M/10 ml.

when \(d\) = the difference between the logs of the duplicate specific activity determinations and \(n\) = the number of duplicates to be compared in an individual experiment; using duplicate determinations of formate incorporation into RNA adenine, RNA guanine and DNA thymine in simultaneous control and 6-MP-treated replicate incubations, \(n = 6\). Comparison of the logs of the means of duplicate incubations were tested by estimating \(t\) by the formula

\[
t = \frac{\log x - \log y}{Sd/\sqrt{2} \times \sqrt{1/2 + 1/2}}
\]

and probability (\(p\)) using \(n-1\) degrees of freedom.

**Results**

**Reproducibility**

To evaluate the variability of the in vitro effect of 6-MP on leukocyte radioformate (C\(^{14}\)) incorporation into the cell nucleic acid bases, antileukemic therapy was withheld in two cases so that repeated studies could be performed. The results are shown in Figure 1. Three studies of the leukocytes of a
### Table 1.—Comparison of Clinical and in Vitro Effects of 6-Mercaptopurine

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Clinical Effect</th>
<th>Clinical Incubation</th>
<th>In Vitro Incubation</th>
<th>Relative Specific Activity</th>
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<td></td>
<td>Control</td>
<td>6-MP- treated</td>
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<tr>
<td>1</td>
<td>Leukopenia</td>
<td>Control</td>
<td>* (.001)</td>
<td>(.001) (.025)</td>
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<td>Pretreatment</td>
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<tr>
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<td>Control</td>
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<td>(.001) (.001)</td>
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<td></td>
<td>(not 6 MP-</td>
<td>6-MP- treated</td>
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<td>0 0.052</td>
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<tr>
<td></td>
<td>resistant)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Leukopenia</td>
<td>Control</td>
<td>0.022</td>
<td>(ns) (ns) (ns)</td>
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<td>1.842 1.077</td>
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</table>

**ACUTE MYELOBLASTIC LEUKEMIA**

**ACUTE LYMPHOBLASTIC LEUKEMIA**
patient with untreated acute myeloblastic leukemia (AML) done within a 15-
day period showed some variation in C¹⁴ incorporation into the nucleic acid
bases in the absence of 6-MP but, in each study, there was a similar and
significant (p < .001) diminution by in vitro 6-MP of C¹⁴ incorporation into
RNA adenine and guanine, while DNA thymine synthesis was unaffected.
Serial studies of the leukocytes of a patient with acute lymphoblastic leukemia
(ALL), begun 2 weeks after the interruption of a 5-month course of therapy
with 6-MP, also yielded similar results. In the first three of four experiments
done in the ensuing 4 weeks, C¹⁴ incorporation into RNA adenine in the
absence of 6-MP showed a gradual increase of doubtful significance; C¹⁴
incorporation into RNA guanine of the control incubations was about the same
in the first three experiments but was greater in a fourth, while incorporation
into DNA thymine was only slightly in excess of background radioactivity in
all four studies. In the experiments in which incorporation in the presence and
absence of 6-MP was compared, there was either no change (expt. 1) or a
slight increase (expt. 4; p < .025) in the amount of C¹⁴ incorporated into cell
RNA adenine and guanine in the presence of the drug.

The leukocytes of a total of 23 cases of acute leukemia were studied in
subsequent experiments. The results in 13 cases were unevaluable: in four
cases death occurred before completion of an adequate clinical trial of 6-MP,
and in the other nine there was insufficient C¹⁴ incorporation into the leuko-
cyte purines to assess the effect of 6-MP in vitro. The results in the remaining
10 cases, grouped according to the morphologic type of leukemia and the
patients' clinical response to 6-MP, are shown in Table 1. In each study more
than half of the incubated leukocytes were blasts. Six of the patients had
myeloblastic (Cases 1–6) and four had lymphoblastic leukemia (Cases 7–10).
Acute Myeloblastic Leukemia

An antileukemic effect of 6-MP, manifested by the development of leukopenia during therapy, was observed clinically in all five of the patients with AML studied prior to therapy (Cases 1-5). Of these, in vitro 6-MP diminished pretreatment C\(^{14}\) incorporation into leukocyte RNA adenine and guanine in three (Cases 1, 2 and 5) and slightly decreased incorporation into DNA thymine in two (Cases 1 and 5). The leukocytes of Case 2 were studied before and after a 5-month course of 6-MP, which was discontinued before the development of clinical drug-resistance, with somewhat different results. Prior to therapy, C\(^{14}\) incorporation into RNA adenine was substantially diminished by 6-MP in vitro, while incorporation into RNA guanine and DNA thymine was insignificantly affected; in the second study, done several weeks after therapy was discontinued, there was complete inhibition of C\(^{14}\) incorporation into both RNA adenine and guanine and a significant decrease in incorporation into thymine.

A study of the leukocytes of Case 5 after clinical resistance to 6-MP developed showed that C\(^{14}\) incorporation into the RNA purines was many times that of the pretreatment values, while incorporation into DNA thymine was relatively unchanged; in vitro 6-MP retained a slight suppressive effect, of borderline statistical significance, on C\(^{14}\) incorporation into both the RNA purines and DNA thymine. Somewhat similar results were observed in the leukocytes of Case 6, which were studied only after clinical resistance to 6-MP had developed.

Acute Lymphoblastic Leukemia

In the leukocytes of the four cases of ALL, C\(^{14}\) incorporation into the RNA purines was either unchanged or enhanced by 6-MP in vitro in a fashion unrelated to the patients' clinical response to the drug. Therapy with 6-MP produced leukopenia in Cases 7 and 8. Prior to treatment, C\(^{14}\) incorporation into the leukocyte RNA purines of Case 7 was slightly increased and incorporation into thymine decreased in the presence of 6-MP in vitro, while C\(^{14}\) incorporation by the cells of Case 8 was unchanged by 6-MP. Cases 9 and 10 were both clinically resistant to 6-MP. The leukocytes of Case 9 were studied during a relapse which followed a 6-MP-induced complete remission. In these cells, C\(^{14}\) incorporation into the RNA purines was significantly increased by in vitro 6-MP and incorporation into thymine was unaffected. In Case 10, leukocytes obtained prior to a course of therapy with 6-MP which produced no evident clinical effect showed no significant C\(^{14}\) incorporation into RNA adenine and no effect of in vitro 6-MP on C\(^{14}\) incorporation into either RNA guanine or DNA thymine.

Discussion

Formate incorporation into the leukocyte nucleic acids is, at best, a gross estimate of de novo purine and thymine synthesis. It reflects only the final steps in the formation of these nucleic acid bases and, in the case of purine synthesis, is dependent on available supplies of 5- amino-4-imidazole car-
boxamide, a precursor requiring only the addition of formate carbon for completion of the purine ring.4 Despite these limitations, the methods used in the present investigation yielded reproducible results when repeated studies were possible and, based on these data, limited conclusions concerning the biochemical effects of 6-MP in human leukocytes seem justified.

In general, the results show no relationship between in vitro suppression of purine synthesis by 6-MP and the antileukemic effects of the drug. Thus, although the leukocytes of seven of the cases of acute leukemia were presumably 6-MP-sensitive, because therapy with the drug produced leukopenia in vivo, RNA purine synthesis was inhibited by 6-MP in vitro in only three. Conversely, in vitro 6-MP suppressed RNA purine synthesis by the leukocytes of two cases in which the disease had become drug-resistant clinically.

These findings are inconsistent with the results of many similar studies in 6-MP sensitive and resistant bacteria and murine tumor cells, in which inhibition of cell growth by 6-MP has been correlated with suppression by the drug of reactions required either for the synthesis of the purine ring or for the interconversion of the completed purines.1,2,7,8 In resistant strains of these cells, unaffected or enhanced purine synthesis in the presence of 6-MP has been attributed to their failure to incorporate 6-MP or to convert it to a metabolically active nucleotide.5,7,8 In the present study, findings consistent with this resistance mechanism were observed in six of the leukocyte populations, four of which were lymphoblastic in type. However, this result was associated with clinical resistance to 6-MP in only two cases, both of lymphoblastic leukemia.

Perhaps reflecting a morphologically related difference in 6-MP-resistance, increased RNA purine synthesis was found in the leukocytes of the two cases of drug-resistant AML which, in one case studied before and after therapy, was acquired in coincidence with clinical resistance to 6-MP. Although in vitro 6-MP retained a significant suppressant effect on RNA purine synthesis in these cells, purine incorporation of formate in the presence of the drug was many times greater than that found in control untreated incubations of the patient's cells before therapy. Increased de novo purine synthesis has also been described in some 6-MP-resistant bacteria,9,10 and may result from a 6-MP-induced increase in cellular reserves of the purine biosynthetic enzymes.11,12 Such a mechanism of resistance would be analogous to the increase in dihydrofolate reductase acquired during the administration of folic acid antagonists.13

Diminution by 6-MP of thymine synthesis by the cells of three AML and one of the ALL drug-sensitive cases, associated in the former with suppression and in the latter with enhancement of RNA purine synthesis, cannot be explained by current concepts of the biochemical actions of 6-MP. These results suggest that in some cells the analog may inhibit the activation of monocarbon units required not only for purine synthesis14 but for thymine synthesis as well, or that changes in RNA purine synthesis may be secondary to an effect of 6-MP on DNA metabolism.

The variety of results obtained in these different leukocyte populations and their lack of correlation with the clinical effects of 6-MP may indicate only that
alterations in nucleic acid synthesis found after 6 hours of incubation with the analog inaccurately reflect earlier biochemical events, perhaps involving the intermediary metabolism of precursors of the nucleic acid purines. Studies of these intermediates at shorter intervals of incubation are in progress. Finally, but less amenable to study, is the possibility that 6-MP acts primarily on marrow cell progenitors of the circulating leukemic leukocytes.

Summary

1. The effects of added 6-mercaptopurine (6-MP) on the in vitro incorporation of radioformate (C\(^{14}\)) into the leukocyte nucleic acid purines and thymine of six cases of acute myeloblastic leukemia (AML) and four cases of acute lymphoblastic leukemia (ALL) have been compared with the patients' clinical response to 6-MP.

2. In seven cases subsequent therapy with 6-MP produced leukopenia. Of these, in vitro 6-MP diminished leukocyte C\(^{14}\) incorporation into the nucleic acid purines in three cases of AML, had no effect in two cases of AML and one case of ALL, and enhanced C\(^{14}\) incorporation into the leukocyte purines of a second case of ALL. Thymine synthesis was slightly diminished by 6-MP in the AML leukocytes in which purine synthesis was inhibited, and in one drug-sensitive ALL leukocyte population in which purine synthesis was slightly increased by the analog. Thus, no regular and consistent relationship between the antileukemic effect of the drug and suppression of purine or thymine synthesis by 6-MP in vitro could be demonstrated.

3. Studies of the leukocytes of two cases of drug-resistant ALL yielded results similar to those observed in presumably drug-sensitive ALL cells.

4. The leukocytes of two cases of AML with acquired drug-resistance showed an increased capacity for in vitro RNA purine synthesis; in the one case in which studies before and after the development of resistance were possible, this property was apparently acquired during therapy. This suggests that an increase in purine biosynthetic enzyme reserves may be a mechanism of 6-MP-resistance in some human leukemias.

Summary in Interlingua

1. Esseva comparate le effectos del addition de 6-mercaptopurina super le incorporation in vitro de radioformato (C\(^{14}\)) ad in le purinas e le thymina del acido nucleic del leucocytos in 6 casos de acute leucemia myeloblastic e 4 casos de acute leucemia lymphoblastic con le responsa clinic del patientes a 6-mercaptopurina.

2. In 7 casos le subequente therapia con 6-mercaptopurina produceva leucopenia. Inter istos, 6-mercaptopurina in vitro diminueva le incorporation leucocytic de C\(^{14}\) ad in le purinas del acido nucleic in 3 casos de acute leucemia myeloblastic, habeva mulle .effecto in 2 casos de acute leucemia myeloblastic e un caso de acute leucemia lymphoblastic, e promoveva le incorporation de C\(^{14}\) ad in le purinas leucocytic de un secunde caso de acute leucemia lymphoblastic. Le synthese de thymina esseva levemente reducite per 6-mercaptopurina in le leucocytos de acute leucemia myeloblastic in le quales le synthese de purina esseva inhibite e in un population de
leucocytes a acute leucemia lymphoblastic con pharmaco-sensibilitate in que le synthese de purina esseva levemente augmentate per le analogo. Assi nulle relation regular e uniforme inter le efecto antileucemic del pharmaco e le suppression del synthese de purina o de thymina per 6-mercaptopurina in vitro poteva esser demonstrate.

3. Studios del leucocytes in 2 casos de pharmaco-resistente acute leucemia lymphoblastic produceva resultatos simile a illos observe in presumitemente pharmaco-sensibile cellulas a acute leucemia lymphoblastic.

4. Le leucocytes in duo casos de acute leucemia myeloblastic con acquirite pharmaco-resistentia manifestava un augmentate capacitate a synthetisar purina da acido ribonucleic in vitro. In un caso, le sol in que studios ante e post le disveloppameimto del resistentia esseva effectuate, iste propriete esseva acquirite apparentemente durante le therapia. Isto suggestiona que un augmento in le reservas de enzymas biosynthetic pro purina es possibilemente un mechanismo del resistentia contra 6-mercaptopurina in certe leucemias human.

REFERENCES


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