Effects of Cytoxan on the Proteins of Sensitive and Resistant Strains of the L1210 Leukemia*

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SUMMARY

Carcinostatic doses of Cytoxan were administered to two groups of mice: one bearing a sensitive form of the L1210 leukemia, the other a resistant form. The incorporation of methionine-S35 into protein was investigated in each of these two groups and in untreated controls. In the resistant tumor there was no significant decrease in methionine incorporation following therapy. In the sensitive tumor there was a marked decrease, which was greatest in the nuclear fractions. No effect of Cytoxan was seen in the livers of any of the animals.

These data indicate that the inhibition observed in the formation of tumor proteins following the administration of alkylating agents is related to the biologic action of these compounds and may be directly related to the mechanism of their activity.

In previous investigations, it has been shown that certain alkylating agents inhibit the incorporation of amino acids into the proteins of the Walker tumor in rats (13). A pattern of inhibition, in which the labeling of nuclear proteins was suppressed to a greater degree than that of cytoplasmic proteins, was observed with a variety of mustards and a number of amino acid tracers. The decrease in amino acid incorporation paralleled a carcinostatic effect on the tumor. However, the alkylating agents are all highly reactive compounds capable of prompt reaction with a variety of compounds found in mammalian tissues. It is, therefore, difficult to establish that biochemical effects observed following the administration of these agents are related to the mechanisms of biologic action. Assessment of these factors was made possible by the development by Lane of a strain of the L1210 leukemia which is resistant to cyclophosphamide (Cytoxan) (11). This permits a comparison of the effects of this alkylating agent in tumors which are biologically sensitive or resistant to its action.

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These experiments were designed to investigate the effects of Cytoxan on the incorporation of amino acids into the proteins of the sensitive and resistant leukemic cells in vivo. After the growth of the tumor was established, a single injection of half the LD50 dose of Cytoxan was given intraperitoneally. Four days later, the tumor-bearing mice were given injections of S35-labeled methionine and its incorporation into the protein fractions of various tissues was studied. It was found that the administration of Cytoxan produced significant inhibition of the incorporation of methionine into the proteins of the sensitive tumor, whereas this process in the resistant tumor was unaffected by Cytoxan treatment.

MATERIALS AND METHODS

The animals used in these experiments were male CDBA mice weighing approximately 20 gm. each. They were obtained from Microbiological Associates, Inc. The leukemia was carried as an ascites in B1D2F1 mice obtained from the Jackson Memorial Laboratory. DL-Methionine (Volk) had an initial specific activity of 40 mc/mmole, and appropriate corrections were made for isotopic decay. The instruments used for measurement of radioactivity (Nuclear Chicago Corporation) recorded 1 μc as 7 X 106 counts/min.

The L1210 leukemia has been carried as an ascites tumor in DBA2 and CDBA mice. Meta-
bolic studies initially performed with the leukemia in this form yielded extremely variable results, concomitant with variability in contamination with red cells and debris. It did not appear that this was a suitable system for quantitative studies. Similarly, following subcutaneous implantation in the abdominal wall, the animals died of leukemia with little or no tumor at the site of injection. In the attempt to grow these cells as homogeneous, solid tumors, variations were made in the techniques and sites of injection. Solid subcutaneous tumors were produced by the injection of 1 ml. of a 1:10 dilution in Hanks solution (37° C.) of the ascitic fluid from leukemic mice into the subcutaneous tissue of the neck. A 35-gauge, 1-inch needle was used for this purpose to facilitate the distribution of the cells over a large area. The tumor is well circumscribed, and, grossly, little or no necrosis has been observed. Microscopically, there are sheets of tumor cells with a minimum of interstitial tissue and rare necrosis. The leukemia grown in this form provides a reproducible system for metabolic studies.

Four days after implantation, the animals were given 250 mg/kg (0.5 LD<sub>50</sub>) of Cytoxan<sup>1</sup> intraperitoneally. Four days later each of the tumor-bearing mice was given an intraperitoneal injection of 6 μc/100 gm of methionine-S<sup>35</sup>. One hour after the injection of the tracer the animals were killed; the tumor and liver were removed, placed in cold 0.25 M sucrose and transferred immediately into the cold room. One-gram samples of tumor and liver were homogenized and separated into nuclear, mitochondrial, microsomal, and cytoplasmic supernatant fractions as previously described (13). The histones were extracted from the nuclear fraction by stirring 8–10 hrs. in 0.25 N HCl. That portion of the nuclear fraction insoluble in HCl was further separated into alkali-soluble (HCl-2) and alkali-insoluble (HCl-1) proteins with 0.1 N NaOH (6). The various fractions were precipitated with perchloric acid (13), and the proteins were isolated, plated, and assayed for radioactivity as previously described (5).

RESULTS

Tumor.—The effects of Cytoxan on the incorporation of methionine into the proteins of the sensitive tumor are shown in Table 1. A decrease was observed in the specific activity of each of the cellular fractions. Statistically significant inhibition of protein labeling followed Cytoxan treatment in all but the HCl-1 and the microsomal fractions. The pattern of inhibition was similar to that seen in previous experiments with other mustards, reflecting a prominence of effects on nuclear proteins. The specific activities of the proteins of the microsomes and mitochondria in treated animals were 79 and 57 per cent of control values: that of the supernatant cytoplasmic proteins was 46 per cent of control; and those of the histone and nuclear HCl-2 fractions were decreased to levels of 47 and 33 per cent of control values. The decrease that occurred in the HCl-2 fraction was, therefore,

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1110 Cy-S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>140 ± 14</td>
<td>71 ± 9***</td>
</tr>
<tr>
<td>Microsomes</td>
<td>209 ± 21</td>
<td>165 ± 3</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>148 ± 15</td>
<td>82 ± 12**</td>
</tr>
<tr>
<td>Supernatant</td>
<td>240 ± 17</td>
<td>115 ± 14***</td>
</tr>
<tr>
<td>Histones</td>
<td>118 ± 6</td>
<td>55 ± 5***</td>
</tr>
<tr>
<td>Nuclear HCl-1</td>
<td>19 ± 2</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>Nuclear HCl-2</td>
<td>136 ± 15</td>
<td>45 ± 5***</td>
</tr>
<tr>
<td>L1110 Cy-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>76 ± 8</td>
<td>93 ± 17</td>
</tr>
<tr>
<td>Microsomes</td>
<td>104 ± 12</td>
<td>191 ± 21</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>77 ± 10</td>
<td>97 ± 13</td>
</tr>
<tr>
<td>Supernatant</td>
<td>157 ± 14</td>
<td>161 ± 22</td>
</tr>
<tr>
<td>Histones</td>
<td>57 ± 7</td>
<td>55 ± 8</td>
</tr>
<tr>
<td>Nuclear HCl-2</td>
<td>64 ± 9</td>
<td>61 ± 8</td>
</tr>
</tbody>
</table>

In this and the subsequent table the data presented are the mean specific activities of protein (counts/min/mg ± the standard error). The data for the treated animals were compared with controls, using the "t" test (*P < 0.05; **P < 0.01; ***P < 0.001). greater than that observed in any other fraction, and statistical significance (P < 0.05) could be shown when compared with the microsomes.

In the resistant tumor (Table 1), treatment with Cytoxan did not result in an inhibition of the incorporation of methionine into proteins. The slight changes in the nuclear fractions were not statistically significant.

Liver.—The effects of Cytoxan on the incorporation of methionine into the proteins of the livers of animals bearing sensitive and resistant tumors are shown in Table 2. In no instance was inhibition observed. In some of the cytoplasmic fractions, an increase in specific activity followed

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1 Generously provided by Drs. E. A. Hawk and W. Nicholl of Mead Johnson and Company.
treatment. However, none of these differences was statistically significant. An increase in activity in the liver following mustard treatment was also found by Bijvoet and Busch (1) in studies of uracil-2-C\textsuperscript{14} incorporation into RNA in animals treated with uracil mustard.

*Protein labeling in the absence of treatment.*—The specific activities of the proteins of untreated sensitive and resistant tumors may be compared in Table 1. The sensitive tumor was considerably more efficient in the formation of protein from methionine. The incorporation of methionine into the untreated, resistant tumor proteins approximated 50 per cent of that incorporated into the proteins of the sensitive tumor. Biologically, the growth patterns of the sensitive and resistant tumors did not differ in untreated animals. Both the sensitive and resistant groups succumbed to the disease on the 10th or 11th day after implantation. Furthermore, differences were not observed in the incorporation of methionine into the proteins of the livers of untreated animals bearing sensitive or resistant tumors. These observations suggest biochemical alteration in the entry or utilization of amino acids that is concomitant with the development of Cytoxan resistance.

**DISCUSSION**

Studies of the mechanism of action of the alkylating agents are complicated by the very high reactivity of these molecules. These agents react with little discrimination with a wide variety of compounds found in biologic systems (16) and produce a variety of pharmacologic effects (14). It is difficult to relate biochemical effects to the mechanisms of biologic action, and the results of many early experiments reflect the use of high concentrations in *vitro*. On the other hand, biological effects of the alkylating agents on the formation of the proteins studied are related to the observed inhibition of tumor growth.

Inhibition by alkylating agents of the formation of proteins was also observed in studies of the incorporation of amino acids into the whole cellular proteins of staphylococci (7) and a lymphosarcoma (15). The recent demonstration by Brookes and Lawley (2) of alkylation on the N-7 position of guanine has provided direct evidence of the reaction of mustards with nucleic acids in biologic systems. These data do not, however, rule out the possibility of cross alkylations between nucleic acids and proteins (18) or a direct effect on nuclear proteins which might alter the synthesis of nucleic acid. Alternatively, an alkylated nucleic acid might influence the synthesis of proteins.

These experiments may also relate to the mechanism by which mustard resistance develops. Incorporation of methionine into the proteins of the

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**TABLE 2**

**EFFECTS OF CYTOXAN ON THE INCORPORATION OF DL-METHIONINE-S\textsuperscript{35S} INTO PROTEINS OF LIVER**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>L1810/Cr-S</th>
<th>L1810/Cr-R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td></td>
<td>No. Counts/min/</td>
<td>No. Counts/min/</td>
</tr>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>6 306 ± 44</td>
<td>6 350 ± 27</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>6 439 ± 51</td>
<td>6 615 ± 32</td>
</tr>
<tr>
<td>Supernatant</td>
<td>6 277 ± 55</td>
<td>6 389 ± 42</td>
</tr>
<tr>
<td>Histones</td>
<td>6 312 ± 41</td>
<td>6 353 ± 33</td>
</tr>
<tr>
<td>Nuclear HCl-2</td>
<td>6 200 ± 20</td>
<td>6 281 ± 23</td>
</tr>
</tbody>
</table>

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resistant tumor was only half as efficient as that observed in the sensitive tumor. Treatment with HN2 has been reported (17) not to affect the transfer of methionine into tumor cells. A greater metabolic rate in the sensitive tumor could provide increased exposure to the active form (3, 8) of the drug. However, the biological behavior of the two tumors was identical in the absence of treatment. Pool sizes could differ in the sensitive and resistant tumors. However, it seems more likely that permeability is altered in the development of resistance, as observed by Nichol and Welch (12) in the case of resistance to amethopterin. These factors are currently under investigation with the use of C14-labeled Cytoxan.

REFERENCES


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