On the Mechanism of Action of the Alkylating Agents

II. Effects of Nitrogen Mustard, Myleran, and X-Radiation on Nucleic Acid Biosynthesis*

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SUMMARY

The effects of nitrogen mustard, Myleran, and x-radiation on the incorporation of isotopic orotic acid, aspartic acid, formaldehyde, adenine, and phosphate into the nucleic acids of the L1210 ascites leukemia are reported. The lack of uniformity in the incorporation pattern following pretreatment with each of the three drugs was considered significant. The treatments could result in stimulation as well as inhibition of precursor utilization. It was concluded that the alkylating agents attack numerous sites with varying degrees of specificity.

In some of the earlier studies, from the marked effect of alkylating agents or irradiation on the P32 incorporation into DNA, it was inferred that nucleotide synthesis or polymerization of nucleotides was the primary target of these agents. More recent investigations, however, showed that the effects of radiation or of alkylating agents are apparently far more complex than originally thought. Davidson and Freeman, for instance, observed an apparent stimulation of P32 incorporation into DNA by HN2, even though there was no significant depression of radiophosphate incorporation into DNA by triethylenemelamine (TEM), triethylenephosphoranfide (TEPA), and triethylenthiophosphoramide (thioTEPA). Heidelberger and Keller reported that methylbis (β-chloroethyl)amine (HN2) stimulated P32 incorporation into nucleotides, whereas the same drug strongly inhibited the utilization of formate for purine biosynthesis in RNA and DNA. On the other hand, Skipper et al. reported little inhibition of formate incorporation into purines by HN2. Drysdale et al. in a recent publication confirmed earlier reports that in vivo mustards produced a more pronounced inhibition of DNA than of RNA synthesis. These authors concluded, however, that there was no evidence that HN2 uniquely inhibits DNA metabolism. Bettendorf et al. (9) reported marked discrepancies between the various isolated nucleotides when P32 incorporation was measured.

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He observed that HN2 produced an apparent decrease in adenosine-5-monophosphoric acid (AMP) formation while there was a stimulation in UTP (5'-uridylic acid) and TMP (5'-thymidylic acid) synthesis. In a recent paper, Shields and Gordy (16) concluded that radicals produced by irradiation of DNA or RNA are different from those of their constituents and suggest that one cannot predict the radiation effects on the nucleic acids from studies of the mononucleotides. Lerman and Tolmach compared the effects of x-rays and HN2 on the DNA polymer (13). In their model they return to the concept of the unstable phosphotriester formation by alkylation agents as proposed by Stacey et al. (20), although, on theoretical grounds, they considered it doubtful whether alkylation per se plays any role in the inactivation of DNA.

In recent years a number of observations were reported which deal with the effects of alkylation agents on protein synthesis. Goodlad confirmed earlier reports of the esterification of amino acid carboxyl groups in proteins (10). Spector et al. observed inhibition of methionine incorporation into proteins by HN2 (17), and similarly Busch et al. (5, 6) showed evidence that an aminouracil mustard had marked effects on protein synthesis in vivo. In our laboratory, Nadkarni also confirmed an earlier observation that the effect of TEM on protein synthesis is more pronounced than that on DNA synthesis. The results of the investigation reported here are thought to be in support of the hypothesis that the mechanism of action of the alkylation agents is based on interference with protein synthesis rather than on the direct inactivation of DNA.

In this paper the effects of Myleran, HN2, and x-radiation on the incorporation of various precursors into RNA and DNA of the L1210 leukemia are reported.

**MATERIALS AND METHODS**

Male DBA2 mice, 10–12 weeks old, were used throughout the experiment. The L1210 A ascites leukemia was transferred by an intraperitoneal inoculation of 10⁶ cells. Periodic total cell counts were made and the ascites tumor was removed for total cell count and the residue sedimented in the cold. The cells were washed 2 times with saline to remove unbound isotope and were then extracted once with cold 10 per cent trichloroacetic acid (TCA) and 4 times with cold 5 per cent TCA. Isolation of the RNA, DNA, fat, and protein fractions was carried out, and the specific activities of the various fractions were estimated by methods described previously (21). The specific activities and the amounts of isotopic material injected were as follows: phosphate-P³² (Tracerlab), 1.83 μC/μM, 71,000 counts/min/mouse; adenine-8-C¹⁴, 0.085 μC/μM, 90,000 counts/min/mouse; formaldehyde-C¹⁴ (Cal. Fdt. Biochem. Res.), 2.65 μC/μM, 725,000 counts/min/mouse; orotic acid-6-C¹⁴ (Tracerlab), 0.95 μC/μM, 1,080,000 counts/min/mouse; dL-aspartic acid-4-C¹⁴ (Tracerlab), 0.76 μC/μM, 880,000 counts/min/mouse.

The following dosage schedules were employed in the treatment of the mice: In each experiment, the animals were divided into four groups of eight animals each. One group received 0.025 mg/mouse of HN2 (Mustargen, Merck), one group received 0.1 mg/mouse of Myleran (Burroughs Wellcome), and one group received 300 roentgens at 275 kv, 0.5 mm. Cu, at 86 r/min. Uniformity of irradiation dose was ascertained by placing the animals singly in irradiation cages which were arranged concentric around the beam. A fourth group of mice served as controls and received saline injections instead of drug or irradiation.

**RESULTS**

The effects of nitrogen mustard (HN2).—As shown in Table 1, HN2 produced a very marked inhibition of isotope incorporation into nucleic acids in most experiments. The amounts of the various isotopic materials in the acid-soluble pool strongly suggest that, although the precursors passed freely into the ascites cells, their further utilization for the de novo synthesis of polynucleotides was inhibited by the drug. This inhibition was most marked in the failing of the cell to incorporate adenine-C¹⁴ into either DNA or RNA. There was a striking discrepancy in the utilization of orotic acid-C¹⁴ under the influence of the drug; namely, a marked inhibition of its incorporation into DNA while there was little if any effect on its utilization for RNA synthesis. The mechanisms for the incorporation of labeled formaldehyde or
aspartate were only moderately affected by this agent.

The effects of Myleran.—As in the case of nitrogen mustard, isotope accumulation in the acid-soluble pool again suggested interference with the utilization of the precursors. There is, however, some evidence that this compound may interfere with the transport of phosphate-P$_{32}$ into the cell, since very low levels of this isotope in the acid-soluble pool were obtained. There was a very pronounced stimulation of the incorporation of orotic acid into both RNA and DNA as compared with that in the controls. This is in contrast to a moderate inhibition produced by this alkylating agent with the other precursors studied.

The effects of x-radiation.—The over-all pattern was similar to that observed with either HN$_2$ or Myleran. Ionizing radiation differed from the other two, however, in that it produced a slight stimulation in the incorporation of adenine into the RNA. Similarly, a moderate increase in the utilization of orotate and aspartate was observed in the RNA under the influence of irradiation, whereas with P$_{32}$ a significant inhibition occurred.

**DISCUSSION**

It was evident that HN$_2$, Myleran, and x-radiation appeared to change the labeling of the acid (TCA)-soluble pool. An impairment of transfer mechanisms across cell membranes was probably reflected in the levels of P$_{32}$ attained in the acid-soluble fraction. Generally, however, there was an elevation of radioactivity of the acid-soluble fraction in the treated tumors, which was interpreted as being due to an inhibition of further utilization of the accumulated isotope. There was no correlation between the availability of a precursor as shown by isotope accumulation in the acid-soluble pool and its rate of incorporation into RNA or DNA. For instance, with HN$_2$, decreased orotic acid utilization in DNA synthesis was unrelated to the amount of precursor available in the acid-soluble pool. Likewise, the effects of HN$_2$ and x-rays on the incorporation of adenine into the RNA are not reflections of the labeling pattern of the acid-soluble pool. It was concluded, therefore, that the agents investigated here did not primarily and substantially interfere with membrane transfer mechanisms.

**TABLE 1**

**INCORPORATION OF PRECURSOR ISOTOPE INTO THE ACID-SOLUBLE POOL, RNA, AND DNA**

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Acid-Soluble Fraction (counts/min/mg protein)</th>
<th>RNA (counts/min/µM ribose)</th>
<th>DNA (counts/min/µM phosphorus)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min.</td>
<td>30</td>
<td>90</td>
</tr>
<tr>
<td>Orotic acid-C$^{14}$</td>
<td>188</td>
<td>128</td>
<td>114</td>
</tr>
<tr>
<td>Adenine-C$^{14}$</td>
<td>57</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Aspartic acid-C$^{14}$</td>
<td>25</td>
<td>53</td>
<td>49</td>
</tr>
<tr>
<td>Phosphate-P$_{32}$</td>
<td>116</td>
<td>120</td>
<td>25</td>
</tr>
<tr>
<td>Formaldehyde-C$^{14}$</td>
<td>216</td>
<td>144</td>
<td>91</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orotic acid-C$^{14}$</td>
<td>236</td>
<td>432</td>
<td>246</td>
</tr>
<tr>
<td>Adenine-C$^{14}$</td>
<td>18</td>
<td>37</td>
<td>34</td>
</tr>
<tr>
<td>Aspartic acid-C$^{14}$</td>
<td>35</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Phosphate-P$_{32}$</td>
<td>76</td>
<td>64</td>
<td>20</td>
</tr>
<tr>
<td>Formaldehyde-C$^{14}$</td>
<td>141</td>
<td>82</td>
<td>73</td>
</tr>
<tr>
<td><strong>Nitrogen mustard</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orotic acid-C$^{14}$</td>
<td>18</td>
<td>34</td>
<td>22</td>
</tr>
<tr>
<td>Adenine-C$^{14}$</td>
<td>35</td>
<td>28</td>
<td>20</td>
</tr>
<tr>
<td>Aspartic acid-C$^{14}$</td>
<td>30</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Phosphate-P$_{32}$</td>
<td>143</td>
<td>134</td>
<td>84</td>
</tr>
<tr>
<td><strong>Myleran</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orotic acid-C$^{14}$</td>
<td>324</td>
<td>340</td>
<td>296</td>
</tr>
<tr>
<td>Adenine-C$^{14}$</td>
<td>15</td>
<td>35</td>
<td>32</td>
</tr>
<tr>
<td>Aspartic acid-C$^{14}$</td>
<td>31</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>Phosphate-P$_{32}$</td>
<td>44</td>
<td>68</td>
<td>27</td>
</tr>
<tr>
<td>Formaldehyde-C$^{14}$</td>
<td>180</td>
<td>199</td>
<td>88</td>
</tr>
</tbody>
</table>

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A rather interesting observation, however, was the finding that occasionally the utilization of a precursor for syntheses was markedly enhanced by the treatment. The contrast between the strong inhibition of orotate incorporation into DNA by HN2 and the nearly threefold increase obtained with Myleran was quite striking. An increase of aspartate uptake into RNA was produced by x-rays in a similar but less marked fashion. It is assumed that the assembly process for the DNA or RNA polymer was affected in many locations on the metabolic pathway. A similar conclusion was reached recently by Bollum et al. (4), who studied the effects of x-radiation on enzymes of DNA synthesis in regenerating rat liver. These authors observed that formation of several enzymes may be delayed or prevented, and their data suggest that the dose-response curve for in vivo effects is multiple in nature. In our experiments, the alkylating agents or the radiation in a number of instances had a marked inhibitory effect on a particular synthetic step. There was, however, an increased utilization of isotopic precursor at other sites, and this was postulated to be a compensatory mechanism, evoked by the cell to overcome a drug-induced defect.

It is proposed from the results of this study that a rather large number of mechanisms, most likely enzymatic in nature, can be susceptible to the action of the alkylating agents or that of x-rays. Such attacks on enzymic mechanisms may take place through S-alkylation, as suggested by Roberts et al. (14), or through a number of possible interactions (15) yet to be elucidated adequately. It is then conceivable that susceptible pathways under attack by a single regimen of treatment are eliminated over the period of a number of cellular generations. If a compensatory mechanism (or metabolic by-pass) attains dominance for a particular metabolic process, a drug-resistant cell might evolve. Considerations such as these might strengthen considerably the argument in favor of a multiple-hit therapy or a so-called sequential blocking therapy.

REFERENCES

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