The Effect of Nitrogen Mustard on the Cellular Concentrations of Nucleic Acids in Regenerating Rat Liver

JOHNE. ULTMANN, ERICH HIRSCHBERG, AND ALFRED GELLHORN

(Institute of Cancer Research and Departments of Biochemistry and Medicine, Columbia University, College of Physicians and Surgeons, New York 32, N.Y.)

Numerous publications during the last 6 years have indicated the importance of the nitrogen mustards in clinical and experimental cancer chemotherapy (9, 10, 21, 32, 33, 41, 42). The principal application has been to the treatment of lymphomas and leukemias. Two compounds, methylbis(β-chloroethyl)amine (HN2) and tris-(β-chloroethyl)amine, have been most widely used.

An equally impressive number of investigations have been concerned with some aspect of the mechanism of action of nitrogen mustard. A wide variety of cytotoxic, antimitotic, mutagenic, carcinogenic, and radiomimetic effects have been described (4, 16, 18, 20, 22, 32). From the biochemical point of view, these findings have led to a consideration of the effects of nitrogen mustard on nucleic acid metabolism. Bodenstein and Kondritzer (3) determined the nucleic acid content of successively older amphibian embryos and found that treatment of the embryos with nitrogen mustard completely prevented the normal rise of desoxyribonucleic acid (DNA) content with age but failed to affect the corresponding increase of ribonucleic acid (RNA) content. Lowrance and Carter (35) found depressions in the content of RNA and DNA and in the incorporation of P32 into the DNA fraction of bone marrow, spleen, and thymus in rabbits during the early periods after treatment with nitrogen mustard. Skipper et al. (40) demonstrated that the administration of carcinostatic agents, including nitrogen mustard, to mice reduced the incorporation of formate-C14 into the combined nucleic acid purines of viscera. Goldthwait (23) recently found that HN2 decreased the incorporation of both formate-C14 and adenine-N15 into adenine of DNA of rat intestine to approximately the same extent. In a recent review (16), Dustin presented the general conclusion that radiomimetic substances produce their effect by inhibiting the synthesis of DNA without causing an appreciable inhibition of the synthesis of RNA. Inhibition of nucleic acid synthesis has been proposed as the principal mechanism of action for mustard gas (25) and for x-radiation (1, 26, 29, 36, 39).

In most of these investigations the rate or extent of nucleic acid synthesis was assessed by determining the rate of incorporation of an isotopically labeled precursor. It was of interest to correlate these findings with determinations of the actual levels of both nucleic acids in the “average cells” of tissues with and without nitrogen mustard treatment. It was anticipated that an effective inhibition of synthesis would be reflected in decreased or unchanged concentrations of nucleic acids per cell. The few previous observations on the effect of nitrogen mustard on the content of nucleic acids in various tissues were made on the basis of wet weight of tissue; the recent recognition of changes in the cellularity of tissues under various conditions (28, 37) indicated that analytical data must be expressed on a cell basis to permit adequate interpretation.

Various biological systems characterized by a high mitotic index and a rapid rate of synthesis of nucleic acids may be suitable for studies of this type. Technical considerations militated against the use of a mixture of viscera, as employed by Skipper et al. (40), for a study requiring this determination of cellularity; to speak of an “average cell” in a homogenate containing all the tissues would be misleading.

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† Present address: New York Hospital, New York 21, N.Y.

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constituting this mixture would be meaningless. However, following the work of Higgins and Anderson (27) and Brues and collaborators (5, 6), regenerating rat liver has been widely employed as a model of rapid, non-neoplastic cell proliferation. Landing et al. (34) obtained an inhibition of mitosis in this tissue by nitrogen mustard but presented no data on concomitant biochemical changes. In untreated animals Stowell (43) demonstrated an increase in the nucleic acid concentration in the nucleolus and in the cytoplasm adjacent to the nucleus on the second day following partial hepatectomy, during the time of rapid cell division. The changes in the concentrations of nucleic acids per cell in the early period of liver regeneration were further defined and placed on a quantitative basis by Price and Laird (37). In the present investigation the observations of Price and Laird have been confirmed and extended to include the effect of nitrogen mustard on the cellular concentrations of nucleic acids during the period of liver regeneration. These experiments had a twofold purpose: (a) to evaluate regenerating rat liver as a model system for studies on the mechanism of action of cancer chemotherapeutic agents and (b) to subject the postulated correlation of inhibition of mitosis and inhibition of nucleic acid synthesis to a different experimental test.

METHODS

Male and female albino rats of the Wistar strain were obtained from our own stock. Animals selected for operation weighed between 180 and 200 gm. Prior to operation the rats were maintained on Purina Laboratory Chow and water ad libitum and were housed in a cooled room at a maximum temperature of 70° F. Following anesthesia with a minimal amount of ether, the rats were partially hepatectomized according to the technic of Higgins and Anderson (27). Nitrogen mustard (methylbis(ß-chloroethyl)amine hydrochloride) (HN2) was administered to the appropriate groups of animals by the technic of Higgins and Anderson (27) and (6) to subject the postulated correlation of inhibition of mitosis and inhibition of nucleic acid synthesis to a different experimental test.

RESULTS

Assessment of hepatectomy.—According to Higgins and Anderson (27) and Brues and Marble (6), 67–75 per cent of the liver was removed in successful partial hepatectomy by their technic. These authors also demonstrated that the average weight of the liver in rats weighing between 125 and 225 gm. is 3.8 per cent of body weight. In the present experiments, the average weight of the liver in eight animals which died during the operation was 3.7 per cent of body weight. The amount of liver removed in 23 animals, taken at random, was calculated to be 68 per cent of the average total liver weight (range 53–86 per cent). It was concluded that our operative procedure was comparable to that of these authors.

Assessment of treatment with HN2.—The most suitable dosage level for subcutaneous administration was chosen on the basis of the work of Anslow et al. (2), Hunt and Philips (31), and Graef et al. (24), who established a dose of 0.9–1.2 mg/kg as the LD50 for administration through the tail vein. It was determined that the concentration of HN2 required for the production of reliable decreases in the white cell count was slightly larger when given by subcutaneous injection. A single dose of 1.9 mg/kg at the time of hepatectomy was found to be accompanied in the first 24 hours by decreases in the white cell count to about 3,000 cells/cmm, but not to lower values; only a few of the rats died during this period. When animals were maintained for 48 and 60 hours, the white cell count frequently dropped below 3,000, and the number of fatalities was greater. The white cell count in untreated rats ranged between 5,000 and 12,000, with an average of 10,000.

In a number of animals, the weight of the spleen was also determined at autopsy to obtain further evidence on the systemic action of HN2. The spleen of untreated animals ranged in weight from 0.55 to 0.76 gm.; in animals treated with HN2, the weight of the spleen ranged from 0.10 to 0.55 gm.
Effect of partial hepatectomy without HN2 treatment.—Table 1 summarizes the changes in cellularity and in the levels of DNA and RNA per cell in regenerating liver. Comparison with the corresponding data of Price and Laird (37) shows excellent general agreement between the results of the two investigations. However, in the present series of experiments the accumulation of DNA did not reach so high a level as in the study by these authors, who reported a rise to 150–180 per cent of control levels at 24 hours after hepatectomy in three separate experiments.

Effect of HN2 on the accumulation of nucleic acids at 36 hours after hepatectomy.—Table 2 summarizes the data obtained in these experiments. The administration of a single dose of HN2 at hepatectomy not only does not interfere with the usual increases in nucleic acid concentration but actually brings about statistically significant increases in the accumulation of both DNA and RNA and a further decrease in the cellularity of the liver 36 hours after the operation.

This conclusion is supported by calculations on the basis of the entire organ. The average weight of the liver 36 hours after hepatectomy was 2.8 gm. in a representative number of control animals and 3.0 gm. in a similar number of treated animals. The average total content of nucleic acids in these livers was slightly higher in the HN2-treated group than in the untreated group (DNA, 5.8 and 5.4 mg/liver, respectively; RNA, 25 and 21 mg/liver, respectively).

The average prehepatectomy values for the cell count in the livers of treated and untreated animals were essentially the same; the average values for DNA as well as for RNA were identical in the two groups. All these values were in close agreement with the average values obtained in the entire series of 106 prehepatectomy livers which constituted the zero-time controls shown in Table 1.

This series of sixteen control rats and 31 rats treated with HN2 was obtained in three separate experiments with four to six control and eight to twelve treated animals each, performed at approximately monthly intervals. The most pronounced differences in nucleic acid levels between treated and untreated animals were found in the first experiment. The same trend was seen in the other two experiments, though the differences between treated and untreated animals were not so great.

In view of the appreciable variation from animal to animal, which will be discussed below, the results obtained in this biological system with small groups of animals are somewhat suspect, and, therefore, statistical treatment of the results was possible only when all three experiments were pooled.

There is a significantly greater accumulation of RNA than of DNA in both treated and untreated animals at 36 hours. This difference in extent of accumulation between the two nucleic acids cannot be explained at the present time.3

TABLE 1

<table>
<thead>
<tr>
<th>Hours after hepatectomy</th>
<th>No. of animals treated or untreated</th>
<th>Cell count (nuclei/mg wet weight)</th>
<th>DNA (μg/cell)</th>
<th>RNA (μg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prehepatectomy</td>
<td>16</td>
<td>164,000 ± 5,500</td>
<td>13.6 ± 0.2</td>
<td>35.9 ± 1.5</td>
</tr>
<tr>
<td>Posthepatectomy</td>
<td>10</td>
<td>131,000 ± 5,100</td>
<td>14.3 ± 0.6</td>
<td>31.1 ± 2.1</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>164,000 ± 5,500</td>
<td>13.6 ± 0.2</td>
<td>35.9 ± 1.5</td>
</tr>
<tr>
<td>HN2 treated</td>
<td>10</td>
<td>131,000 ± 5,100</td>
<td>14.3 ± 0.6</td>
<td>31.1 ± 2.1</td>
</tr>
</tbody>
</table>

The possibility was considered that some of the “orcinol color” in these experiments should be attributed to glycogen rather than to RNA. Preliminary experiments indicated, however, that the addition of glycogen, at concentrations higher than those to be expected in regenerating rat liver, to homogenates prior to the Schneider procedure did not affect the di-phenylamine reaction for DNA and brought about only a small additional “orcinol color.” The magnitude of this possible glycogen blank was not sufficient to account for the significantly greater accumulation of RNA.

TABLE 2

<table>
<thead>
<tr>
<th>Effect of HN2 treatment on cellularity and nucleic acid levels per cell of regenerating liver 36 hours after hepatectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>No. rats</td>
</tr>
<tr>
<td>Cell count (nuclei/mg wet weight)</td>
</tr>
<tr>
<td>DNA (μg/cell)</td>
</tr>
<tr>
<td>RNA (μg/cell)</td>
</tr>
<tr>
<td>Prehepatectomy</td>
</tr>
<tr>
<td>Posthepatectomy</td>
</tr>
<tr>
<td>Prehepatectomy</td>
</tr>
<tr>
<td>Posthepatectomy</td>
</tr>
</tbody>
</table>

\[ D/S_D = \sqrt{\frac{M_1 - M_2}{S_1^2 + S_2^2}} \]
liminary results obtained at these time periods following hepatectomy and administration of the single dose of HN₂ are summarized in Table 3. It would appear that treatment with the drug has no demonstrable effect on the DNA or RNA content of the cells of regenerating liver at these times. The data of Friedenwald et al. (19) on corneal epithelium and of Landing and associates (34) on regenerating liver indicate that inhibition of mitosis after a single dose of nitrogen mustard may be expected to disappear in 36–48 hours.

Control experiments.—It was ascertained in preliminary experiments that there were no differences in the analytical results between portions of the same liver assayed immediately after removal from the animal and after maintenance at —20° C. for variable periods of time. In agreement with Price and Laird (37), it was found that the DNA and RNA values in livers removed 36 hours after laparotomy without hepatectomy showed no appreciable variations from the control values.

The administration of the usual dose of HN₂ to rats after laparotomy or to rats not subjected to any surgical manipulation had the expected effect on the white cell count but brought about no changes in the DNA or RNA levels per cell in livers removed 36 hours after the injection. Physiological saline, when administered to normal, sham-operated, or partially hepatectomized animals, had no effect on the white cell count or nucleic acid values.

Prior to the analysis of liver homogenates from treated animals, it was desired to determine the effect of HN₂ on the diphenylamine and orcinol reactions employed in the nucleic acid estimations. For this experiment, 10 mg. HN₂ was dissolved in 10 ml. phosphate buffer, and the solution was allowed to stand for 1 hour at body temperature to permit cyclization to occur. This solution was diluted with 5 per cent trichloroacetic acid, and aliquots were added to portions of the final 5 per cent trichloroacetic acid extracts prepared from homogenates of normal rat liver. The level of HN₂ in the final extract was about 10 times that permitted 36 hours after laparotomy without hepatectomy. The level of HN₂ in the final extract was about 10 times that of the control values.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>CONTROL</th>
<th>HN₂ TREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. rats</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Cell count (nuclei/mg wet wt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prehepatectomy</td>
<td>174,000</td>
<td>179,000</td>
</tr>
<tr>
<td>Posthepatectomy</td>
<td>157,000</td>
<td>144,000</td>
</tr>
<tr>
<td>DNA (µg/cell)</td>
<td>8.6</td>
<td>10.5</td>
</tr>
<tr>
<td>RNA (µg/cell)</td>
<td>19.4</td>
<td>19.6</td>
</tr>
</tbody>
</table>

The two methods yielded essentially the same picture of the comparative cellularity of these livers, within the limits of error of the two procedures. Qualitative examination of these slides revealed easily detectable differences which are demonstrated in Figures 1–3. The stroma of the regenerating liver is less compact than the control. The cells are larger in the former, and the nuclei are also consistently larger, although they show significantly greater variation in size. The frequency of mitotic figures is high in the regenerating liver sections and virtually absent in the control liver. These findings are in general agreement with the data of Price and Laird (37) and Stowell (48). A comparison of the histological appearance of regenerating liver from nitrogen mustard-treated and untreated animals fails to demonstrate a marked difference. It is our impression that the frequency of mitotic patterns is lower in the former than in the latter.

**DISCUSSION**

Before attempting to interpret the results of the present investigation in terms of the mechanism of action of nitrogen mustard, it is important to evaluate the method by which the results were obtained and the suitability of the biological system, which has been employed.

**Evaluation of the method**.—Several procedures based on entirely different approaches have been

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**TABLE 3**

<p>| EFFECT OF HN₂ TREATMENT ON CELLULARITY AND NUCLEIC ACID LEVELS PER CELL OF REGENERATING LIVER 48 AND 60 HOURS AFTER HEPATECTOMY |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>GROUP</th>
<th>48 Hours HN₂ Treated</th>
<th>48 Hours Control</th>
<th>60 Hours HN₂ Treated</th>
<th>60 Hours Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (µg/cell)</td>
<td>8.6</td>
<td>10.5</td>
<td>9.6</td>
<td>8.9</td>
</tr>
<tr>
<td>RNA (µg/cell)</td>
<td>19.4</td>
<td>19.6</td>
<td>19.7</td>
<td>19.6</td>
</tr>
<tr>
<td>Cell count (nuclei/mg wet wt)</td>
<td>174,000</td>
<td>179,000</td>
<td>208,000</td>
<td>210,000</td>
</tr>
<tr>
<td>Posthepatectomy</td>
<td>157,000</td>
<td>144,000</td>
<td>164,000</td>
<td>188,000</td>
</tr>
</tbody>
</table>

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used for the estimation of the nucleic acid content of various mammalian cell populations. The simplest and least time-consuming method consists of homogenization of a sample, direct enumeration of nuclei in one aliquot of the homogenate, and determination of the nucleic acid content in another aliquot. This approach has been subjected to an extensive investigation in a wide variety of tissues during the last 2 years and has been shown to yield valid and reliable results.¹

In the present series of experiments, the following findings have confirmed the conclusion that this simple method is a trustworthy guide: (a) The average value obtained for the DNA content of normal rat liver in 106 animals was 10.4 ± 0.2 μg/cell (cf. Table 1); this value is in excellent agreement with the values obtained by a variety of other procedures (11, 13, 14, 17, 37), though higher than the value reported by Cunningham and collaborators (12). (b) The accumulation of nucleic acids after hepatectomy in these experiments follows the pattern described by Price and Laird (37) in most respects; the careful evaluation these authors made of the suitability of this method for studies on regenerating liver offers further support for the reliability of the present data. (c) It is realized that if the characteristic decrease in cell count observed in the early periods after hepatectomy were an artifact produced by a supposable fragmentation of nuclei during homogenization, then the increases in nucleic acid concentrations calculated on a cell basis would be entirely illusory. This decrease in cell count was, however, confirmed by the histological evidence which has been alluded to.

Evaluation of the biological system.—Regenerating rat liver offers advantages as well as disadvantages for the investigation of the biochemical mode of action of antitumor agents. This model system of non-neoplastic growth is characterized in its early stages by a mitotic index comparable to that of anaplastic transplanted tumors (6, 44). Moreover, liver regeneration is an unfailing consequence of adequate partial hepatectomy, so that an ample supply of experimental material can be produced at will.

The chief drawback of this system is that, although regeneration never fails to follow hepatectomy, the rate of regeneration, at least from the point of view of nucleic acid synthesis, is subject to considerable variation. This variation may be caused by a number of factors (7, 8, 15, 30 [pp. 82]), among them, the nutritional status of the animals before and after operation, diurnal and seasonal variations in mitotic activity, environmental temperature, strain, age, and weight of the animals, severity of the trauma produced by the operation and concomitant anesthesia, intensity of the humoral stimulation of regeneration, etc. While attempts were made to control these factors in the present experiments, it is recognized that some of them must have continued to operate at least to some extent.

In many experimental situations, variations of this magnitude might not have any crucial significance. The following considerations, however, indicate that they become an important factor in experiments with this tissue.

One of the biochemical processes which must take place in most biological systems before a cell can divide is an increase in its nucleic acid content. A variety of findings (cf. 30 [pp. 101–3]) suggest that the DNA content reaches approximately twice the resting cell level shortly before the actual division of the cell into two daughter cells. In regenerating liver, the cells of the remaining portion of liver following hepatectomy may then be envisaged to prepare for their first mitosis by doubling their DNA content. If all the cells were carrying out this function at exactly the same time, it would be theoretically possible to select a moment after hepatectomy at which the DNA content of the “average cell” would be twice that of the prehepatectomy sample. Such a finding is, of course, highly unlikely in view of the differences in operative trauma, humoral stimulation of regeneration, access to building blocks, synthetic ability, etc., which must exist between cells in different areas of the remaining lobe of the liver. At any one time, some cells will be in the resting stage, others will be carrying on nucleic acid synthesis prior to division, others will be just ready to divide, yet others will have newly arisen by mitosis. The DNA content of the “average cell” at this time will then be some value intermediate between normal and twice normal, and the quantitative range of expression of any effect of a chemotherapeutic agent on this value will be severely limited. Therefore, relatively large numbers of animals must be used in the control and treated groups to permit a statistical evaluation of any change which may be observed, and the significance of the result is not necessarily commensurate with the effort which is required to document it. This practical drawback must be taken into account when the suitability of regenerating rat liver for these studies is assessed.

Effect of HN2 on the nucleic acid content of the “average cell.”—Two conclusions may be drawn from the data which have been presented. First, the administration of nitrogen mustard does not prevent the accumulation of nucleic acids which
normally takes place in the early periods of liver regeneration. The present experiments do not provide any information concerning the rate of this accumulation, but it is clear that at 36 hours after hepatectomy the treated cells do contain less nucleic acid than the untreated cells. Second, the present data actually demonstrate that there is an appreciably greater accumulation of nucleic acids in the treated cells than in the controls. The significance of this finding may be discussed briefly.

In the simplest interpretation, the nucleic acid concentration per cell at any time after hepatectomy may indicate the number of liver cells which have not yet divided for the first time; the higher this value in relation to the concentration before hepatectomy, the larger the number of cells yet waiting to divide. The significantly higher levels of nucleic acids in the “average cell” after nitrogen mustard treatment would then indicate that at 36 hours posthepatectomy a significantly greater number of liver cells have not yet entered the first mitosis. The absence of any demonstrable difference in nucleic acid levels in treated and untreated cells at 48 and 60 hours suggests that cell division is postponed for only a relatively small number of hours when a single dose of the drug is given.

The conclusion that this postponement of mitosis in a significant number of cells is accompanied by a net rise in the nucleic acid content of the “average cell,” i.e., that the cells in which mitosis is blocked already appear to contain the high premitotic level of nucleic acid, is the central result of this investigation. This study cannot provide any direct evidence for or against an inhibition of synthesis of nucleic acid by HN2; it does, however, demonstrate that inhibition of cell division by this agent in regenerating liver is not mediated primarily through such an inhibition of this synthetic process. It may be postulated that HN2 interferes with mitosis either (a) by a mechanism not directly involving the nucleic acids or (b) by blocking nucleic acid utilization; the decreased incorporation of precursor into nucleic acid purines observed by Skipper et al. (40), Goldthwait (23), and others might then be a secondary consequence of the fact that further synthesis would cease once the doubling of nucleic acids in these blocked cells has taken place. The data which have been presented would also be consistent with the assumption that both synthesis and utilization of nucleic acids were interfered with by this drug, but that the inhibition of utilization was significantly more pronounced than the inhibition of synthesis.

It should be stressed that an attempt to correlate incorporation data with formate-C14 obtained in the viscera of mice and static nucleic acid analyses in regenerating rat liver is hazardous at best and that generalizations from any particular system to a variety of tissues would not appear permissible at the present time. Therefore, experiments are now in progress to assess the incorporation of nucleic acid precursors in regenerating liver and the effect of other drugs on the nucleic acid levels in this and other biological systems.

SUMMARY

1. The suitability of regenerating rat liver for studies on the biochemical mechanism of action of cancer chemotherapeutic agents has been evaluated.
2. In agreement with data obtained by other investigators, it was found that there was an accumulation of nucleic acids in the liver cells in the early periods of regeneration following partial hepatectomy. This accumulation reached its peak 36 hours after hepatectomy.
3. The subcutaneous injection of a single dose of nitrogen mustard was followed by a significantly greater accumulation of nucleic acids in the liver cells at 36 hours after hepatectomy. At 48 and 60 hours, there was no longer a demonstrable difference in the nucleic acid levels per cell between treated and untreated animals.
4. It appears that in this biological system the inhibition of mitosis by nitrogen mustard was not mediated primarily through an inhibition of nucleic acid synthesis, as suggested by the work of other investigators with isotopically labeled precursors of nucleic acids.

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