Nitrogen Mustard and Triethylene Melamine Content in Normal and Tumor Tissues after Intra-arterial and Intravenous Injection in Rats

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In recent years a number of reports have indicated that nitrogen mustard exerts a greater effect in certain human neoplasms when administered intra-arterially than when given by the conventional route of injection (1, 2, 3, 11–13). Intra-arterial treatment was originally considered of value primarily in neoplasms confined to the extremities or head. Maxfield and his associates have recently treated more generalized carcinomatosis of the abdominal and thoracic cavities by regional injections of nitrogen mustard and have obtained beneficial results.

There appear to be at least three possible explanations for the increased effectiveness of nitrogen mustard when it is injected intra-arterially rather than intravenously. There may be: (a) an increased selective uptake of the agent by the tumor, (b) a simple increase in concentration of the agent in all the tissues, including the tumor in the region supplied by the injected artery; and (c) an increased tolerance to the drug by the patient which allows the use of larger amounts of nitrogen mustard than may be employed when intravenous administration is used.

The present study was undertaken to determine which, if any, of the above hypotheses could serve to explain the enhanced action of nitrogen mustard when injected intra-arterially, and coupled with this were studies to determine if there were similar increases in the effect of triethylene melamine administered under similar conditions. The degree of localization of these two drugs in various tissues, including neoplasms, was also investigated.

MATERIALS AND METHODS

Adult, male, Sprague-Dawley rats weighing 250–310 gm, were used throughout the study. They were maintained three or four to a cage and given water and Purina chow ad libitum.

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The Walker rat carcinoma 256 was chosen for study of the localization of nitrogen mustard and triethylene melamine in neoplastic tissue, since this particular tumor is sensitive to, though not curable by, these drugs. Transplantation of the tumor was made intramuscularly into the right thigh according to the technic described by Green and Lushbaugh (8).

Intra-arterial injections were made into the abdominal aorta of the rats as follows: The animals were anesthetized with pentobarbital, and a midline incision was made through the lower abdominal wall with clean, but not aseptic, technic. The intestines were retracted and kept moist with saline. Injections were made into the aorta through a 27-gauge needle inserted about midway between the renal and iliac arteries. No attempt was made to dissect the aorta free from the surrounding tissues, since these tissues helped to control bleeding. Following injection of the solutions into the aorta, the needle was left in place for 1 minute to allow the injected solution to be completely transferred from the site of injection. The needle was then removed and light pressure applied to the site to control bleeding, which was minimal in all cases. The peritoneal incision was sutured and the skin closed with wound clips. Intravenous injections were made into the common iliac vein by means of a similar technic. In some instances, intravenous injections were made into the right jugular sinus under direct visualization. In this case, the small incision was closed with a single wound clip. Control animals were subjected to the same operative treatment and given injections of saline.

Methyl-C4-H2-bis(β-chloroethyl)amine hydrochloride and 2,4,6-triethyleneimin-1,3,5-triazine-8,8,8-C14†, hereafter referred to as HN2 and TEM, respectively, were used in the tracer studies. The specific activities of the HN2 and TEM were 14.6 μc/mg and 10.1 μc/mg, respectively. The compounds were assayed for radioactivity by the liquid scintillation method described by Hayes et al. (8).

The relative toxic effect of HN2 and TEM administered by intravenous and intra-arterial injection was determined for a series of graded doses. The doses were 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg HN2/kg body weight, and 0.5, 1.0, 1.25, 1.5, and 2.0 mg TEM/kg body weight. Twelve rats were injected at each dose level.

To compare the lethal action of nitrogen mustard in normal and tumor-bearing animals, rats in which the Walker carcinoma had been transplanted 10 days previously were also given injections. The injections were made into the right femoral artery, just proximal to the tumor. Five tumor-bearing rats were given injections at each dose level. The doses were 1.5, 2.0, 2.5, and 3.0 mg/kg body weight. Survival was followed for 30 days after treatment in all instances.

† The labeled compounds were synthesized in this Laboratory. The HN2 was synthesized by Arthur Murray III (4), and the TEM was synthesized by Lloyd Williams (17).
To determine the concentrations of C14-labeled HN2 or TEM in various tissues following intravenous and intra-arterial administration, groups of three normal rats and three rats bearing a 7-day-old Walker rat carcinoma were given injections of 1.0 mg HN2 or 1.0 mg TEM/kg body weight. The injection volume was kept constant at 0.3 ml. Since it is known that HN2 and TEM react rapidly in the body (10), the animals were sacrificed 5 minutes after injection and the various tissues removed for analysis.

The following tissues were analyzed for C14 content: tumor, whole blood, lung, kidney, gastrointestinal tract, liver, spleen, sternum, femur, femoral marrow, mesenteric lymph nodes, thymus, thigh and back muscle, and testis. The thigh muscle and femoral marrow were obtained from the left hind leg, while the whole femur was removed from the right hind leg. The back muscle was removed from the high thoracic region. A section of the gastrointestinal tract, including a portion of the ileum and colon, was washed free of feces and used for analysis. Blood was obtained by heart puncture at time of sacrifice. Blood and femoral marrow were put directly into small, tared, glass tissue homogenizers and reweighed to give specific weight of the tissue. All other tissues were dropped singly into liquid nitrogen, frozen, pulverized, and then allowed to thaw. Approximately 50-60 mg aliquots of these tissue homogenates were counted in suspension by the liquid scintillation method (8). All counting was done in a Los Alamos 540 coincidence system counter. The results are expressed as 0.1 µg equivalents/gm of tissue (i.e., the tissue radioactivity equivalent to the activity of 0.1 µg of the injected HN2 or TEM).

An attempt was made to correlate histological damage with the concentration of the drugs in the tissues. Normal rats were given injections intra-arterially and intravenously of 1.0 mg HN2 or 0.8 mg TEM/kg body weight and sacrificed for histologic study at intervals of 0.5, 1, 3, 7, and 14 days.

RESULTS

Table 1 compares the effect of intra-arterial and intravenous administration of HN2 and TEM to normal rats at various dose levels. The results are expressed in per cent survival at 30 days. The table also includes the results for tumor-bearing rats which received a series of doses in the femoral artery. These data show that, for normal rats, the same dose of HN2 injected intra-arterially is less toxic than when injected intravenously. However, for tumor-bearing rats injected just proximal to the tumor, the same dose was more toxic than if it were injected intravenously into normal rats. There was no significant difference in the survival of normal rats when treated with TEM at either of the two injection routes at any of the dose levels tested.

Charts 1 and 2 show the daily per cent survival of normal animals for 30 days following intravenous and intra-arterial injection of 1.5 mg HN2 or 1.0 mg TEM/kg body weight. No deaths oc-

| TABLE 1 | PER CENT SURVIVAL* OF RATS FOLLOWING VARIOUS DOSES OF HN2 AND TEM, ADMINISTERED INTRA-ARTERIALLY (IA) OR INTRAVENOUSLY (IV) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| COMPOUND | RECIPIENT RAT | DOSE OF (MG/KG) | ADMINISTRATION | 0.5 | 1.0 | 1.25 | 1.5 | 2.0 | 2.5 | 3.0 |
| HN2 | Normal | IA | 67 | 67 | 14 | 100 |
| HN2 | Tumor-bearing | IA | 58 | 15 | 17 | 0 |
| TEM | Normal | IV | 67 | 0 | 0 | 0 |
| TEM | Tumor-bearing | IV | 0 | 0 | 0 | 0 |

* Survival was followed for 30 days after treatment.
curred later than 10 days after injection; the greatest number of deaths occurred between the 3d and 5th days. These graphs further emphasize the difference in toxicity of HN2 injected intra-arterially and intravenously and the lack of difference for TEM. No mortality was observed in the saline-treated controls.

Distribution of C14-labeled HN2 and TEM.—The distribution of C14 activity from labeled HN2 and TEM in tissues of tumor-free and tumor-bearing rats given injections intravenously and intra-arterially is summarized in Table 2. The data are given as the mean value obtained from a series of three, and are expressed in 0.1-μg equivalents of HN2 or TEM/gm tissue. Differences between the values for each tissue were tested for significance by the “t” test. Those having a P value of 0.05 or less have been indicated in the table.

In tumor-free animals which received HN2, the tissues showing significantly higher amounts of C14 activity following intra-arterial injection as compared with intravenous injection were the femoral marrow, thigh muscle, and the femur. These tissues were all located just distal to the site of injection into the aorta. Tissues showing a significantly lower concentration were the thymus, testes, spleen, liver, and gastrointestinal tract. In tumor-free animals which received TEM, only two tissues differed significantly when the two injection routes were used. They were thigh muscle and femoral marrow, and in both cases the values were higher following intra-arterial than following intravenous injection.

In tumor-bearing rats, a comparison of tissue values from the intra-arterial injection of HN2 with those from the intravenous injections shows only two that are significantly higher—the whole femur and the Walker rat carcinoma 256. The whole femur was obtained from the right leg, in which the tumor was located. However, the values for C14 activity in the femoral marrow, obtained from the left leg, did not differ significantly. Those tissues which had values significantly lower, intra-arterially, than those obtained intravenously, were sternum, thymus, spleen, and gastrointestinal tract. A similar inspection of the values for tumor-bearing animals receiving TEM reveals that only the tumor was significantly higher intra-arterially versus intravenously, with the rest of the

### TABLE 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>HN2</th>
<th>TEM</th>
<th>HN2</th>
<th>TEM</th>
<th>HN2</th>
<th>TEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Back muscle</td>
<td>3.8</td>
<td>3.0</td>
<td>6.3</td>
<td>7.4</td>
<td>5.5</td>
<td>6.6</td>
</tr>
<tr>
<td>Sternum</td>
<td>5.1</td>
<td>7.0</td>
<td>6.3</td>
<td>7.6</td>
<td>6.6</td>
<td>9.4*</td>
</tr>
<tr>
<td>Thymus</td>
<td>6.6</td>
<td>12.5*</td>
<td>8.7</td>
<td>9.2</td>
<td>11.3</td>
<td>14.1*</td>
</tr>
<tr>
<td>Testes (both)</td>
<td>2.9</td>
<td>5.5*</td>
<td>3.1</td>
<td>5.2</td>
<td>3.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>7.3</td>
<td>12.6*</td>
<td>9.0</td>
<td>9.3</td>
<td>8.1</td>
<td>12.8*</td>
</tr>
<tr>
<td>Liver</td>
<td>11.1</td>
<td>15.9*</td>
<td>9.3</td>
<td>9.8</td>
<td>11.3</td>
<td>17.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>59.3</td>
<td>71.6</td>
<td>18.4</td>
<td>21.1</td>
<td>62.3</td>
<td>78.5</td>
</tr>
<tr>
<td>Lung</td>
<td>12.0</td>
<td>15.2</td>
<td>9.9</td>
<td>9.4</td>
<td>15.5</td>
<td>15.8</td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td>11.4</td>
<td>15.8</td>
<td>10.2</td>
<td>9.4</td>
<td>16.5</td>
<td>21.2</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>6.3</td>
<td>9.6*</td>
<td>7.8</td>
<td>7.5</td>
<td>8.9</td>
<td>10.9*</td>
</tr>
<tr>
<td>Thigh muscle (left leg)</td>
<td>5.5</td>
<td>3.2*</td>
<td>10.8</td>
<td>6.2*</td>
<td>4.5</td>
<td>5.4</td>
</tr>
<tr>
<td>Femoral marrow (left leg)</td>
<td>35.3</td>
<td>11.5*</td>
<td>46.4</td>
<td>9.4*</td>
<td>15.3</td>
<td>12.6</td>
</tr>
<tr>
<td>Femur (right leg)</td>
<td>20.7</td>
<td>5.9*</td>
<td>10.8</td>
<td>3.8</td>
<td>13.8</td>
<td>4.5*</td>
</tr>
<tr>
<td>Blood</td>
<td>15.3</td>
<td>12.1</td>
<td>12.1</td>
<td>11.9</td>
<td>8.4</td>
<td>10.6</td>
</tr>
<tr>
<td>Tumor (right leg)</td>
<td></td>
<td></td>
<td>54.3</td>
<td>12.9*</td>
<td>48.2</td>
<td>12.0*</td>
</tr>
</tbody>
</table>

* Values differ significantly (P = 0.05) for the two routes of administration.

Table 3 lists the P values, obtained by “t” testing, for the differences among various combinations of tissue concentrations. The intra-arterial values from tumor-free and tumor-bearing animals receiving HN2 have been compared (column 1), as have the intravenous (column 2). The same has been done for the values from animals receiving TEM (columns 3 and 4). In a similar manner, the intra-arterial values from normal rats receiving HN2 and TEM are compared (column 5), as well as the intravenous (column 6); finally, the same comparisons were made of the values from tumor-bearing rats (columns 7 and 8). Significant differences, when they occur, have been so indicated, and reference to Table 2 will indicate which of the two values being compared is the higher, which the lower.

Of note are the differences found between the
intravenous values of normal animals receiving HN2 and TEM. At least half of the tissues from the HN2 animals, namely, thymus, spleen, liver, lung, kidney, mesenteric lymph nodes, and thigh muscle, are higher in concentration than those from the TEM animals. In contrast, a comparison of the intra-arterial values when the two drugs were injected into normal animals shows the concentration to be higher only in the kidney in the HN2 animals as compared with those receiving TEM, whereas concentrations in the back and thigh muscle are lower.

**Histopathological Studies**

*Nitrogen mustard.*—Sections of femoral and sternal marrow, spleen, thymus, mesenteric lymph node, jejunum, testis, liver, kidney, adrenal, and lung were obtained at intervals of 6 hours, 1, 3, 7, and 14 days after intravenous and intra-arterial injection of HN2. The tissue changes found at these intervals were qualitatively similar to those described in detail by other authors (5). No quantitative differences in the degree of cellular damage were found in animals injected by the different routes except in the case of the bone marrow and testis. These will be the only organs described in any detail.

Degenerative changes in the bone marrow seen at 6 hours were similar for both groups of animals. The changes consisted of early rhexis and pyknosis of hematopoietic or lymphoid cells, regardless of the route of administration.

Sections taken 1 day after injection of HN2 showed considerable hemorrhage in the sternal and femoral marrow with some depletion of cells. Cellular debris usually seen at earlier time intervals had been largely removed.

At 3 days, the degenerative changes had progressed in the bone marrow of the rats injected intravenously. At this time there was a marked loss of cellularity with a "shift to the right" in the cell type (i.e., most of the remaining cells were relatively mature in type). The changes were more pronounced than those seen in the femoral marrow. The situation was reversed in the case of the intra-arterially injected rats in that the femoral marrow showed a greater degree of destruction than did the sternal marrow.

At 7 days, maximum differences in the sections from intravenously and intra-arterially injected rats were seen. Sections of sternal marrow (Figs. 1 and 2) from both groups showed a considerable degree of regeneration with regenerative changes more nearly complete in sections from the rats receiving HN2 intra-arterially. The femoral marrow (Figs. 3 and 4), on the other hand, showed less evidence of regeneration in animals injected intra-arterially, while these changes were fairly far advanced in sections from the intravenously injected group.

Sections taken at 14 days showed the sternal marrow from both groups to be very hyperplastic, as was the femoral marrow from the intravenously injected group. In the case of the intra-arterially injected rats, the femoral marrow had not completely regenerated.

The differences in degree of cellular damage found in the testes of the animals injected by the different routes were quite variable from animal to animal as well as between the testes of the same...
animal in some instances. In general, the degenerative changes following intra-arterial injection were dependent upon the location of the injection site relative to the arterial blood supply to the testis. Degenerative change in cells in all phases of maturation occurred as early as 1 day, regardless of the route of injection. There was little change in this picture on the 3d day. By the 7th day, many of the animals showed sloughing of the germinal epithelium and many multinucleated cells in the lumina of the tubules. Sections taken on the 14th day still showed considerable denudation in some sections, while others showed evidence of considerable repair of the germinal epithelium.

No differences in the pathological picture were noted when the C14-labeled HN2 was used in the place of nonlabeled HN2, or when the route of intravenous injection was by jugular sinus or by common iliac vein.

**TEM.**—Histopathological studies following TEM administration were similar to those described for HN2, with the exception that no striking differences were seen in the bone marrow following the two different routes of administration.

**DISCUSSION**

Damage to the bone marrow and/or the gastrointestinal tract is generally believed to be the limiting factor in nitrogen mustard therapy (9). Following intravenous injection of C14-labeled HN2, there was a fairly uniform distribution of the C14 activity in all marrow-containing bones and approximately equal destruction of marrow in all areas. Intra-arterial administration of labeled HN2 produced an unequal distribution pattern of C14 activity. Since a portion of the marrow and other rapidly proliferating tissues, such as thymus, spleen, and gastrointestinal tract, were relatively protected by intra-arterial as opposed to intravenous injection, intra-arterially administered HN2 should be less toxic. The toxicity experiment showed that this was the case, since there was a good correlation between the distribution and toxicity studies.

In tumor-bearing rats, the intra-arterial administration of HN2 increased the toxicity of the drug. This could be owing to the increased uptake of HN2 by the tumor and the consequent extensive necrosis of tumor cells. While more HN2 is localized in the tumor following regional arterial injection, care should probably be exercised in applying this procedure clinically. Perhaps the drug should be administered at lower doses and over a long period of time in order to decrease the toxic effect of tumor cell destruction.

Correlation of C14 activity in tissues with histopathologic changes following labeled HN2 administration was not particularly successful. This is not surprising, since the quantitative histological evaluation of severity of damage is a crude estimate at best, and it is not likely that small variations in concentration would produce detectable differences in histological appearance.

Occasionally, there were apparent discrepancies between the tissue concentrations of C14 and the severity of histopathological damage. Several explanations may account for these discrepancies. In the sternum, there was no significant difference in C14 concentration following intravenous and intra-arterial administration, but the sections from intra-arterially injected animals showed less marrow damage than did those receiving the drug intravenously. Since it was not possible to obtain an adequate marrow sample, the entire sternum was assayed for C14 activity, which resulted in an abnormally low amount. The effect of analyzing the whole bone as opposed to the marrow alone is shown by comparison of C14 activity in the femur with that of the femoral marrow.

Testicular damage following intra-arterial injection was extremely variable. Repeated studies showed that the C14 activity following intra-arterial injection of labeled HN2 and the extent of testicular damage were critically dependent on the point of injection into the aorta. Anomalous branching of the internal spermatic arteries from the aorta (7) or variations in back pressure at the time of injection of the HN2 could easily result in wide differences in C14 concentration in the testes of the animal, and even in difference between the testes in the same animal.

Comparison of C14 concentrations following the two routes of administration of labeled HN2 and TEM revealed marked differences in tissue distribution.

Tissues just distal to the intra-arterial injection site of HN2 showed C14 activity 2–4 times as high as that found in similar tissues from animals injected intravenously. Many of the tissues beyond the first capillary bed to the intra-arterial injection site showed uniformly lower concentrations. These differences were particularly pronounced in rapidly proliferating tissues such as thymus, testis, spleen, liver, and gastrointestinal tract.

Wide differences in tissue distribution following intravenous and intra-arterial injection were not seen following the administration of TEM-C14. The only tissues showing a significant difference...
in C\textsuperscript{14} activity between the two routes of administration were the femoral marrow, thigh muscle, and tumor. Concentrations in these tissues were higher following intra-arterial injection. This relatively equal distribution of TEM, regardless of the route of injection, would also account for equal toxicity whether the drug was injected intra-arterially or intravenously.

Based on these results, it may be concluded that tissue fixation was more rapid in the case of HN\textsubscript{2} than in the case of TEM. Since tissue concentrations of TEM (as measured by C\textsuperscript{14} activity) were little influenced by the route of administration, it seems probable that TEM may complete one or more passages through the circulatory system before becoming fixed. More rapid fixation of HN\textsubscript{2} must occur because of the marked influence of the route of administration on tissue concentrations. This interpretation must be viewed with some reservation, because the actual concentrations of these compounds were not measured (16). Rather, the C\textsuperscript{14} concentrations were determined. It is believed, however, that such determinations provided a reasonably accurate index of the extent to which these compounds reacted in the various tissues, since both compounds react rapidly with tissue and the determinations were made on tissues obtained 5 minutes after injection. Had a longer time interval been employed, the measurements of C\textsuperscript{14} activity might have been an indication of the concentration of metabolic breakdown products. These could have been translocated from one tissue to another (14–16) as a function of time.

In conclusion, it appears that intra-arterial therapy of neoplasms with HN\textsubscript{2} offers the following advantages over intravenous therapy: (a) higher concentrations are obtained in the tumor and other tissues just distal to the site of injection; and (b) slightly higher total dosages may be tolerated. Regional intra-arterial administration of TEM produces a higher concentration in the tumor than does intravenous administration, but the toxicity of this compound is not influenced by the route of administration. There appears to be less experimental justification for the clinical use of intra-arterial injections of TEM than for HN\textsubscript{2}.

SUMMARY

The toxicity, histopathology, and tissue distribution of nitrogen mustard (HN\textsubscript{2}) and triethylene melamine (TEM) were studied following intra-arterial and intravenous injection into normal and tumor-bearing rats. Intra-arterial injection of C\textsuperscript{14}-labeled HN\textsubscript{2} resulted in higher C\textsuperscript{14} activity in tissues just distal to the injection site. Intra-arterial injection of TEM had little effect on its toxicity or on the tissue distribution of C\textsuperscript{14} activity from labeled TEM. Both drugs showed higher concentrations of C\textsuperscript{14} activity in tumor tissue following intra-arterial administration proximal to the tumor. Fixation of HN\textsubscript{2} in tissues appeared to be more rapid than that of TEM. There appears to be less experimental justification for the clinical use of intra-arterial injections of TEM than for HN\textsubscript{2}.

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![Figure 1](cancerres.aacrjournals.org) Rat sternal marrow 7 days after intravenous injection of 1.0 mg HN\textsubscript{2}/kg body weight. Eosin-azure II\times320.

![Figure 2](cancerres.aacrjournals.org) Rat sternal marrow 7 days after intra-arterial injection of 1.0 mg HN\textsubscript{2}/kg body weight. Eosin-azure II\times320.

![Figure 3](cancerres.aacrjournals.org) Rat femoral marrow 7 days after intravenous injection of 1.0 mg HN\textsubscript{2}/kg body weight. Eosin-azure II\times320.

![Figure 4](cancerres.aacrjournals.org) Rat femoral marrow 7 days after intra-arterial injection of 1.0 mg HN\textsubscript{2}/kg body weight. Eosin-azure II\times320.

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