Methods of Altering Nitrogen Mustard Toxicity in Dogs. I. Spleen and Mesenteric Artery Exclusion

By Neil Lempert, Robert P. Leather and William B. Scharfman

There are many points of similarity between the response of tissues to ionizing radiation and to alkylating agents. Particularly notable is the great sensitivity of hematopoietic tissues to both; in fact, this sensitivity is the main factor in limiting the total tolerated dosage of either of these agents. In 1949 Jacobson demonstrated that shielding the spleen of mice protected against the lethal effects of total body irradiation. This phenomenon is not completely explained, but is probably due to repopulation of the bone marrow by uninjured hematopoietic cells from the spleen, rather than to humoral substances liberated by the uninjured spleen. These observations have not been duplicated to date in larger animals.

When alkylating agents are infused into the circulation it is possible to exclude different anatomic areas from the effects of these drugs by preliminary cross-clamping of the blood supply. Since these agents are fixed relatively quickly by the tissues, 90-95 per cent tissue-fast in three minutes, the isolation of extremities, or visceral segments, can be accomplished without risk of devitalization. These characteristics of nitrogen mustard make it particularly suited to the experiments undertaken in our study. For example, it has been shown by Houck that occlusion of the mesenteric vasculature for 15 minutes spared the intestinal mucosa from the usual pathologic changes attended by HN₂ administration. Similarly, cross-clamping the aorta for 2 minutes spared the femoral and lower vertebral bone marrow. Work in our laboratory has shown that the vessels in the splenic pedicle and the superior mesenteric artery may be occluded with vascular clamps for 20 minutes without gross or histologic evidence of damage to the spleen or small intestine.

We were therefore interested in determining whether the spleen would act as a marrow donor in dogs by temporarily excluding it from the circulatory bed during the period of HN₂ fixation in tissues. Further, we wished to determine whether exclusion of the intestines would augment a protective effect of splenic exclusion. If such protection could be demonstrated, the safe dosage range of HN₂ and other alkylating agents might thereby be extended.

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"HN₂," and "mustard" apply colloquially throughout to nitrogen mustard (methyl bis B-chloroethyl amine hydrochloride).
METHODS

Experiments were performed on 47 apparently healthy, mongrel dogs (selected from colony cages). All animals were anesthetized with intravenous Nembutal and intubated with endotracheal tubes. Each animal received 600,000 units of penicillin and 10 cc. of anti-distemper vaccine daily, for 15 days following mustard administration. All were checked for vomiting and diarrhea as well as for signs of pneumonia and distemper daily. The animals were given food and water ad libitum and intravenous fluids when decreased fluid intake, diarrhea, vomiting or dehydration were noted. Complete blood counts including platelets and reticulocytes were done at least every other day and often daily. Bone marrow aspirations were performed in the initial experiments and were then abandoned because it was undesirable to anesthetize nitrogen mustard-treated dogs in the postoperative state and we experienced considerable difficulty obtaining satisfactory marrows without anesthesia.

Spleen exclusion was accomplished in all cases by cross-clamping the splenic pedicle using two vascular clamps so that the pulse to the spleen was completely obliterated for 8 to 10 minutes. The mesenteric artery was occluded in a similar manner. In all experiments, pulsations to the spleen and mesentery were restored with release of the clamp. At this time, in all cases the occluded tissue appeared viable.

The entire experiment was subdivided into multiple groups: two animals were initially studied testing the efficacy of shielding the spleen from the general circulation with the use of RISA (radio-iodinated human serum albumin). After first obtaining blood from the peripheral circulation and splenic vein for background counts, the spleen was occluded, 10 mc. of RISA were injected into a peripheral vein and samples were collected every 5 minutes for 20 minutes from peripheral venous blood and splenic venous blood. These were counted for 2 minutes in duplicate and compared to the control counts. The following 45 experiments were then performed at varied dosages of nitrogen mustard with control and experimental animals, as indicated in table 1. Dog weights varied between 25 and 40 pounds. In all cases the nitrogen mustard was freshly prepared by dilution of 10 mg. nitrogen mustard with 5 cc. of normal saline 10 minutes before usage. The nitrogen mustard was injected into intravenous tubing attached to a #18 gauge needle inserted into a hind leg vein. The patency of the needle and evidence of non-infiltration were insured in all cases. All control animals were anesthetized but were not subjected to laparotomy.

An animal living 30 days following injection of nitrogen mustard was considered a survival. Animals in the series were then submitted to complete autopsy, including all survivors sacrificed at 60–90 days. At this time specimens of spleen, bone marrow and individual organ pathology, if noted, were obtained.

RESULTS

In the initial studies testing the efficacy of splenic shielding with the use of vascular clamps, both animals injected with RISA showed no appreciable entrance of radioactive material into the splenic vein after 15 minutes of occlusion. This was felt to substantiate the effectiveness of the technic used for excluding the spleen from the vascular bed. Furthermore, routine hematoxylin and eosin studies of 10 spleens following cross-clamping of the pedicle failed to reveal the presence of significant damage, with the exception of one animal. Ten spleens examined from control animals (spleen not excluded) showed cellular destruction, loss of architecture and, later, fibrosis in seven instances. Of the remaining three animals, two survived for 60 days and showed only minimal fibrosis.

The following results were obtained in 45 experiments as shown in table 1.
ALTERING HN₂ TOXICITY IN DOGS I

Table 1

<table>
<thead>
<tr>
<th>HN₂ Dose mg./Kg.</th>
<th>Control</th>
<th>Spenic Exclusion</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No. of Dogs</td>
<td>Lived</td>
</tr>
<tr>
<td>0.8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>1.0</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>1.2 with mesenteric artery exclusion</td>
<td>20</td>
<td>5</td>
</tr>
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1. Five dogs, two of which were spleen-shielded, received 0.8 mg./Kg. of nitrogen mustard with no mortality in any animal.

2. Nine animals, four of which were spleen-shielded, received 1.0 mg./Kg. of mustard. Of this group, two shielded and three control dogs died. (1.0 mg./Kg. has been previously reported by Anslow as the LD₅₀.)

3. Thirty-one dogs received 1.2 mg./Kg. of nitrogen mustard. In this group 13 were spleen-shielded with 10 dogs receiving simultaneous mesenteric artery occlusion. Of the 18 control animals, 10 also had simultaneous mesenteric artery occlusion. The results indicate that 7 of 13 spleen-shielded dogs and 11 of 18 control dogs died before 30 days. When the mesenteric artery was clamped, 10 of the 20 dogs survived.

An analysis of the complete blood counts, including platelet and reticulocyte counts, on these 45 animals shows that with initial intravenous administration of nitrogen mustard there is a transient rise in the white blood count as well as hematocrit and hemoglobin. At approximately 2 to 4 days the reticulocyte count reaches zero, and at 4 to 7 days there is a moderate decrease in platelets. Only an occasional animal in this series manifested bleeding that could possibly be attributed to a platelet deficiency, and this was always in the form of bloody diarrhea. Bleeding per rectum, however, is a poor means of clinically measuring platelet deficiency since nitrogen mustard has a known toxic effect on the gastrointestinal mucosa in dogs. The white blood count seemed most susceptible to nitrogen mustard, however, and there was always a marked diminution in white cell count in approximately 4 to 7 days.

As indicated in figures 1 and 2, neither spleen-shielding nor mesenteric artery shielding appears to alter significantly the white cell depression due to the nitrogen mustard. Also, the differential count during these days usually showed a marked preponderance of lymphocytes. Of those animals surviving the initial insult, the white blood count as well as the platelets and reticulocytes usually returned to normal or higher than normal values by the fifteenth day after HN₂ administration. There is no evidence from these experiments that more rapid regeneration of bone marrow, as indicated by the white blood cell count, occurs with spleen-shielded animals. Postmortem examination of the bone marrow of those animals, both shielded and unshielded, dying within 8 days of nitrogen mustard administration, showed marked marrow depression. All dogs surviving for at least 10 days following nitrogen mustard administration exhibited bone marrow regeneration.
All groups showed the major cause of death to be either acute pneumonia or distemper with pneumonitis. Fluid and electrolyte imbalance was felt to be an important factor in two deaths. Four dogs were noted with gangrene of the small and large bowel. Three of these animals were controls and one had both mesenteric and splenic shielding.

**DISCUSSION**

Recently, a number of experiments in animals have been described in an attempt to utilize the spleen in the prevention of marrow depression. Demetz, working with nitrogen mustard in rabbits, showed that survival is not increased when the splenic pedicle is occluded, or when intraperitoneal homologous splenic homogenates are administered. Survival was improved, however, by splenectomy prior to administration of mustard. The explanation for this result is at the moment unknown. Longerbeam and Lillehei, working with supralethally irradiated dogs, demonstrated consistently improved survival rates with the administration of autogenous splenic homogenates. They were, however, unable to demonstrate any protection with spleen shielding.
Fig. 2.—Splenic and mesenteric vascular shielding \((\text{HN}_2 1.2 \text{ mg./Kg.})\).

Sullivan\textsuperscript{15} et al., doing similar work with dogs, were unable to show an increased survival with splenic homogenates. These two studies are of particular interest because in Sullivan's study the highest total dose of splenic cells was 2.7 billion whereas Lillehei's group was able to attain a dose of 5.9 billion cells. It would therefore appear that the total cell dosage may be a very important factor in reducing mortality after supralethal irradiation of dogs. It has been previously reported that smaller animals such as the mouse are protected against supralethal irradiation by a variety of spleen and bone marrow preparations.\textsuperscript{1,2} Ellinger has described the successful use of cell-free splenic extracts to enhance survival in guinea pigs.\textsuperscript{16}

Bone marrow, of course, is the ideal substance with which one can repopulate a depressed marrow. Experiments using autogenous marrow in irradiated dogs have been described by a number of authors and are best exemplified by work from Thomas' laboratory\textsuperscript{17} showing increased survival with autogenous marrow. Subsequent work from this same laboratory showed benefit in some instances from homologous marrow transplants following total body irradiation and employing meticulous animal techniques.\textsuperscript{16,27} Although iso-
lation units and a kennel-raised stock of dogs are very desirable and no doubt increase the number of survivors, we feel that they are not mandatory and, indeed, such conditions are very difficult to attain in most laboratories.

At the clinical level, various attempts at preventing marrow depression have been described. The most frequently used technic has been the harvesting of marrow from a patient prior to therapy with intravenous replacement of this autogenous marrow after drug administration or body irradiation.\textsuperscript{19-23} These technics are difficult to evaluate because of varying dosages and the lack of control material. Bierman et al.\textsuperscript{24} have recently described "leukapheresis" (fractionated white cell concentrates) in conjunction with occlusive arterial tourniquets in treating tumor patients with nitrogen mustard. Using these methods the authors report the use of doses of nitrogen mustard up to 4.0 mg./Kg. Conrad and Crosby\textsuperscript{5} have utilized arterial tourniquets on all four extremities and obtained 100 per cent survivors using 1.5 mg./Kg. of nitrogen mustard. They point out that the LD\textsubscript{50} dose of mustard is 1.0 mg./Kg. in humans.

In a recent report, two malignant tumors were treated with splenectomy, regional perfusion of the tumors with nitrogen mustard, followed by infusion of the autogenous splenic homogenate. The homogenate contained 30 billion cells.\textsuperscript{26} However, no conclusion could be made from these two patients except that the technic was possible and could be performed safely. With this background one can readily anticipate the possibilities for utilizing the spleen as a means of repopulating a depressed marrow.

From the experiments described in this paper it would appear that marrow toxicity cannot be prevented by obliterating the splenic vessels so that the spleen is shielded from the effects of the drug. It has been demonstrated by Lillehei that if enough cells are given by homogenizing the spleen, protection may be afforded the animal subjected to lethal x-ray. The reason for this failure to protect, with intact shielded spleen, would seem most likely to be due to the failure of adequate cellular ejection from the spleen into the circulation to permit repopulation of the marrow to take place effectively. Furthermore, the humoral explanation for bone marrow protection is now made even more untenable. We intend to investigate this problem further by using cell-free splenic extracts in nitrogen mustard-treated dogs.

The x-ray protection in Jacobson's original mouse experiments is best explained by bone marrow repopulation which is effective in the mouse but not in larger animals. In addition, the spleen may be a more important factor hematopoietically in a smaller species.

Previous observers have commented on mesenteric vascular shielding as a means of decreasing gastrointestinal toxicity with mustard. Our experiments confirm this fact and similarly do not demonstrate a change in over-all mortality, although a possible beneficial effect cannot be excluded.

At present, work is now in progress in our laboratory to investigate the effectiveness of autogenous and isologous splenic homogenates as well as cell-free splenic extracts in the prevention of nitrogen mustard toxicity.
ALTERING \( \text{HN}_2 \) TOXICITY IN DOGS

SUMMARY AND CONCLUSIONS

Forty-five dogs have been treated with varying dosages of nitrogen mustard. Nineteen of these dogs were subjected to cross-clamping of the splenic vessels prior to administration of the drug. No appreciable improvement in mortality or degree of marrow depression has been found with this technic.

Exclusion of the spleen and small intestine has been found to be a practical and reliable method for protecting these organs from the effect of intravenous administration of nitrogen mustard.

SUMMARIO IN INTERLINGUA

Quaranta-cinque canes esseva tractate con varie doses de mustarda de nitrogeno. Dece-novem esseva subjicite a crampage cruciate del vasos splenic ante le administration del pharmaco. Nulle appreciabile melioration esseva obtenite per iste technica, tanto in le mortalitate como etiam in le grado de depression del medulla.

Esseva trovate que exclusion del splen e del intestino tenue es un practic e certe metodo pro proteger iste organos ab le effectos del administration intravenose de mustarda de nitrogeno.

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