# Effects of Alkylating Drugs and Combinations of X-Irradiation and Cortisone on Tumor Immunity *

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OUR INTEREST in the effect of alkylating drugs on tumor immunity began when we treated a series of patients with advanced cancer with triethylenethiophosphoramide (TSPA).[1] Some patients seemed to deteriorate rapidly, as though a defense mechanism, which was holding the tumor in check, was impaired by the drug.

The literature contains information on the destructive effect of alkylating drugs on immunity to bacteria [8] and on the effects of nitrogen mustard in lowering host resistance to homologous tissue transplants and thus promoting the success of skin grafts [5] and renal transplants.[2]

With increasing use of alkylating drugs as adjuncts to surgery in patients with visceral cancer, the effects of these drugs on host-tumor relationships may be important, although host-tumor relationships themselves are poorly defined.

The investigation reported in this paper was undertaken to determine if two alkylating agents can alter immunity to a transplantable tumor. Combinations of x-irradiation and cortisone are known to be effective in conditioning otherwise resistant animals to transplants of tumors,[17] therefore, a comparison was made between these agents and the alkylating drugs as to their effects on tumor immunity.

## Methods

The tumor used was the Bagg lymphosarcoma, which has been maintained in our laboratory for more than 225 consecutive transplants. After subcutaneous inoculation into young male rats of the Wistar strain, the tumor grows progressively and causes death in 83 per cent of animals. In the other 17 per cent, the tumor grows into a mass measuring 1 × 1 × 1 cm. or more and then regresses.

Bagg tumor cells were obtained by excising a tumor from a subcutaneous site. The tumor was minced so as to liberate individual tumor cells. A suspension of these cells in human serum was made, and the suspension was filtered through a fine mesh wire screen. Care was taken to avoid bacterial contamination. The number of living tumor cells in each suspension was determined by a staining technic which differentiates living from dead cells.[12] The method for the preparation of living cells for inoculation into animals has been described in detail previously.[10]

Between May, 1957, and September, 1958, 218 male Wistar rats which had survived growth and regression of the tumor were given a second subcutaneous inoculation consisting of two to 13.4 million living tumor cells in 0.1 ml. of suspension. No tumors developed in these animals indicating that the animals had become re-

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TABLE 1. *Alteration of Induced Immunity to Bagg Lymphosarcoma (as determined by growth of subcutaneous tumor implants)*

| | Treatment Prior to Tumor Implant | Subsequent Challenges: Number of Rats | Rats with Takes* | Rats Killed by Tumor |
|---|---|---|---|---|
| Immune Rats | None (Controls) | 119 | 0 | 0 |
| | Various doses $HN_2$** | 91 | 0 | 0 |
| | Various doses TSPA† | 41 | 0 | 0 |
| | Various doses x-irradiation and cortisone‡ | 118 | 26(22%)§ | 10(8%) |

* A take is a solid growth of tumor 1 cm. or more in its smallest diameter.

** See Table 2.

† Twenty-one rats had single doses of 1 to 6 mg./kg; 20 had multiple doses totalling 6 to 17 mg./kg. Tumor cells were given 1 to 15 days after the last dose of TSPA.

‡ See Table 3.

§ Significantly greater than the control percentage ($P < .01$).

sistant to the tumor and thus had acquired an induced immunity.

The alkylating agents used were mechlorethamine hydrochloride (nitrogen mustard) ($HN_2$) and triethylenethiophosphoramide (TSPA). The drugs were given intraperitoneally.

Animals to be irradiated were placed in a wooden container with separate compartments. Eight animals were given whole-body irradiation at one time using a 220-KV machine at 15 milliamperes at a distance of 95 cm. Filtration was with 0.5 mm. Cu plus 1 mm. Al (HVL 1 mm. Cu). The time of exposure was 12 to 16 minutes depending on the dose. The dose rate was 12.5 r./min.

Following x-irradiation, cortisone ** was given subcutaneously in the thigh or in the nape of the neck in divided doses of 5 to 20 mg./day at two day intervals. The entire treatment was completed in seven days.

### A. Effect of Alkylating Drugs as Compared to X-Irridation and Cortisone on Induced Immunity

Three hundred and sixty-nine male Wistar rats with an induced immunity to Bagg tumor were used in this experiment. Weights at the beginning of the experiment were 200 to 350 Gm. One hundred and ninteen animals remained untreated to serve as controls. Ninety-one were treated with $HN_2$, 41 with TSPA, and 118 received x-irradiation and cortisone. Prior to the experiment, most of the animals were given one or more subcutaneous challenge doses of the tumor, and in each case the tumor failed to grow, thus verifying that the animals were immune. After receiving treatment, the rats were again given a subcutaneous inoculation of tumor into the flank. Observations were made of the subsequent growth of tumor to determine if the immunity had been altered by the treatment.

Total doses of $HN_2$ varied from 0.6 to 6.0 mg./kg. in single or multiple doses as indicated in Table 2. Doses of 1.6 mg./kg. or more caused weight loss and depression of leucocytes. The $LD_{50}$ for a single dose of $HN_2$ is 1.0 to 2.0 mg./kg. for the rat.[8] The dose of $HN_2$ suitable for administration to patients as an adjuvant to surgery for cancer of the lung is 0.3 or 0.4 mg./kg. in divided doses over a two or three-day period.

Doses of TSPA ranged from 1.0 to 17 mg./kg. When more than 6 mg./kg. was

** Cortisone acetate (11-dehydro-17-hydrocorticosterone-21-acetate) (Upjohn).

TABLE 2. *Doses of $HN_2$ Given to Tumor-Immune Rats*

| Doses of $HN_2$ mg./kg. Prior to Tumor Implant | Subsequent Challenges: Living Tumor Cells × $10^6$ | Days After Last Dose of $HN_2$ | Number of Rats | Rats with Takes | Rats Killed by Tumor |
|---|---|---|---|---|---|
| 0.6 | 3.2 | 7 | 8 | 0 | 0 |
| 0.6 | 13.4 | 6 | 11 | 0 | 0 |
| 1.6 | 13.4 | 6 | 11 | 0 | 0 |
| 2.4 (0.4, 3 × weekly for 2 weeks) | 6.0 | 1 | 16 | 0 | 0 |
| 3.6 (0.4, 3 × weekly for 3 weeks) | 6.0 | 3 | 15 | 0 | 0 |
| 4.8 (0.4, 3 × weekly for 4 weeks) | 2.0 | 3 | 10 | 0 | 0 |
| 6.0 (0.4, 3 × weekly for 5 weeks) | 6.0 | 3 | 20 | 0 | 0 |
| Totals | | | 91 | 0 | 0 |

given, a divided dose schedule was used. Single injections of 4 mg./kg. or larger caused weight loss. Respiratory infections were common in animals given 1.0 mg./kg. at two or three-day intervals when the injections were continued for two weeks or more. Doses of TSPA recommended for adjuvant cancer chemotherapy in patients are 0.6 to 0.8 mg./kg. in divided doses given over a three or four-day period. Thus, the doses of alkylating drugs varied from amounts which produced no toxic effect to the maximum that the animal could tolerate and still remain in good condition.

X-irradiation and cortisone were given according to the schedules listed in Table 3. These doses were also well tolerated by the animals and were toxic but not lethal.

Subcutaneous inoculations of tumor cells were given at intervals after the last injections of $HN_2$ or TSPA as indicated in Tables 1 and 2, or on the same or next day after completing the course of x-irradiation and cortisone. The number of living cells injected varied from two to 13.4 million for control animals and for those treated with $HN_2$, and was three to 11.5 million for animals treated with TSPA. Six million cells were given to the x-irradiated and cortisone treated animals. There is no difference in tumor growth in normal animals resulting from inoculations containing cells within this dose range.

In these experiments, induced immunity to Bagg lymphosarcoma was not altered with $HN_2$ or TSPA, but in 22 per cent of animals treated with x-irradiation and cortisone the immunity was broken down sufficiently to allow growth of tumor, and in eight per cent the tumor was fatal (Table 1).

TABLE 3. *Doses of X-Irradiation and Cortisone Given to Tumor-Immune Rats*

| Doses Prior to Tumor Implants: X-ray (r.) | Cortisone (mg.) | Subsequent Challenges (6 × $10^6$ Living Cells): Number of Rats | Rats with Takes | Rats Killed by Tumor |
|---|---|---|---|---|
| 150 | 40 (10 mg. × 4) | 14 | 8 | 0 |
| 150 | 60 (15 mg. × 4) | 33 | 4 | 2 |
| 200 | 60 (15 mg. × 4) | 56 | 12 | 8 |
| 200 | 80 (20 mg. × 4) | 15 | 2 | 0 |
| | Totals | 118 | 26 | 10 |

TABLE 4. *Growth of Bagg Tumor in Lewis Rats*

| Prior Treatment | Subcutaneous Challenges ($2.8 \times 10^6$ to $6.0 \times 10^6$ living tumor cells) | | |
|---|---|---|---|
| | Number of Rats | Rats with Takes | Rats Killed by Tumor |
| | | Per Cent | |
| Males | | | |
| None (Controls) | 37 | 97 | 41 |
| $HN_2$* | 17 | 100 | 47 |
| X-irradiation + cortisone** | 23 | 100 | 96† |
| Females | | | |
| None (Controls) | 42 | 100 | 14 |
| $HN_2$* | 16 | 94 | 19 |
| X-irradiation + cortisone** | 25 | 100 | 96† |

* Doses of $HN_2$ were 2.4 mg./kg. (0.4 mg. 3 × weekly for 6 doses) or 3.6 mg./kg. (0.4 mg. 3 × weekly for 9 doses).

** Doses were 200 r. plus 20 to 80 mg. of cortisone in divided doses (5 to 20 mg. for 4 doses).

† Significantly greater than the control percentage ($P < .01$).

### B. Effect of $HN_2$ as Compared to X-Irradiation and Cortisone on the Resistance of Lewis Rats to Bagg Lymphosarcoma

An inbred strain of Lewis rats, maintained in our laboratory by brother-sister mating, was used for this experiment. When the Bagg tumor is transplanted subcutaneously in these animals, growth of the tumor occurs in almost all animals of both sexes. Approximately 40 per cent of male rats die from a progressively enlarging tumor. In female rats the tumor usually regresses, and most females survive. Rats less than eight months old were used. Weights of males were 100 to 400 Gm. Females were 100 to 300 Gm. An attempt was made to alter the resistance of these animals to the tumor. Doses of $HN_2$ were 2.4 to 3.6 mg./kg. Doses of x-irradiation were 200 r and cortisone 20 to 80 mg. Immediately following this treatment, six to 7.5 million living Bagg tumor cells were injected subcutaneously into the flank. There was no significant alteration of the susceptibility of these animals to the tumor as a result of treatment with $HN_2$. In contrast, x-irradiation and cortisone lowered the animals' resistance to the tumor and increased its lethal effect (Table 4).

### C. Effect of $HN_2$ as Compared to X-Irradiation and Cortisone on the Cytotoxicity of Immune Rat Serum and Immune Rat Lymphocytes against Bagg Lymphosarcoma

We have previously shown that serum and lymphocytes of Wistar rats with an induced immunity to Bagg lymphosarcoma are cytotoxic to cells of this tumor. The cytotoxicity of serum was demonstrated by *in vitro* [13-15] and *in vivo* [9, 10] methods, and, more recently, the cytotoxicity of lymphocytes was demonstrated by *in vivo* experiments.[10, 11] Thus, both humoral and cellular antibodies play a part in this tumor immune mechanism. Serum [9] and lymphocytes [11] from normal (nonimmune) rats have no effect on this tumor.

In this experiment, serum and lymphocytes of rats with an induced immunity were tested to determine if these elements retained cytotoxic properties against Bagg lymphosarcoma after the animals had been treated with x-irradiation and cortisone.

Immune male rats weighing 250 to 350 Gm. were divided into two groups. The first was treated with $HN_2$ or with x-irradiation and cortisone, and the second remained untreated to provide controls.

Immediately after treatment, rats of both groups were anesthetized with nembutal and bled by cardiac puncture. The serum was separated by centrifugation. Thymus glands and spleens were removed from the animals, and suspensions of lymphocytes were prepared from these organs in the same manner as was used for the preparation of Bagg tumor cells.[10] The number of living cells was determined in each suspension. One milliliter of suspension was used for injection.

TABLE 5. *Effect of Treatment of Immune Rats on the Cytoxicity of their Lymphocytes (as determined by intraperitoneal inoculation of tumor cells plus immune lymphocytes into normal rats)*

| Treatment of Immune Rats | Intraperitoneal Inoculum into Normal Rats | | Normal Rats | | |
|---|---|---|---|---|---|
| | Number of Lymphocytes from Immune Rats × $10^7$ | Number of Bagg Tumor Cells × $10^5$ | Total Inoculated | Surviving 23 Days | |
| | | | | No. | % |
| $HN_2$ | | | | | |
| 2.4 mg./kg. (0.4 × 6) | 1.4–16 | 7 | 11 | 9 | 82* |
| 3.6 mg./kg. (0.4 × 9) | 5.6–6.8 | 9 | 22 | 18 | 82* |
| X-ray + cortisone | | | | | |
| 150 r. + 40 mg. | 1.0–48 | 7–10 | 16 | 15 | 94* |
| None | 20–63 | 7–10 | 60 | 47 | 78* |
| | None | 7–10 | 150** | 26 | 17 |

* Significantly greater than the control percentage ($P < .01$).
** Controls.

$HN_2$ and the x-irradiation and cortisone combination caused atrophy of the lymphoid organs. The spleens of animals treated by both methods were approximately one-fourth the size of untreated immune animals and one-half the size of normal nonimmune rats of the same age, sex and size. Even greater decreases in the size of the thymus glands resulted from this conditioning.

An average of 2.7 ml. of serum was obtained from animals treated with $HN_2$ and 2.6 ml. from x-irradiated and cortisone treated animals. Six ml. of serum was the average amount obtained from untreated immune rats. Therefore, it was apparent that there was a decrease in blood volume of treated as compared to untreated immune animals.

The sources of the serum and lymphocytes were 41 immune rats conditioned with $HN_2$, 23 conditioned with x-irradiation and cortisone and 40 untreated immune rats.

Serum and lymphocytes from treated and untreated immune animals were inoculated intraperitoneally into normal male Wistar rats immediately following an in-

TABLE 6. *Effect of Treatment of Immune Rats on the Cytotoxicity of Their Serum (as determined by intraperitoneal inoculation of tumor cells plus immune serum into normal rats)*

| Treatment of Immune Rats | Intraperitoneal Inoculum into Normal Rats | | Normal Rats | | |
|---|---|---|---|---|---|
| | Serum from Immune Rats | Number of Bagg Tumor Cells × $10^5$ | Total Inoculated | Surviving 23 Days | |
| | | | | No. | % |
| $HN_2$ | | | | | |
| 2.4 mg./kg. (0.4 × 6) | 2 ml. | 7–10 | 25 | 21 | 84* |
| 3.6 mg./kg. (0.4 × 9) | 2 ml. | 9 | 10 | 10 | 100* |
| X-ray + cortisone | | | | | |
| 150 r. + 40 mg. | 2 ml. | 7–10 | 21 | 20 | 95* |
| None | 2 ml. | 7–10 | 80 | 64 | 80* |
| | None | 7–10 | 150** | 26 | 17 |

* Significantly greater than the control percentage ($P < .01$).
** Controls.

traperitoneal inoculation of tumor cells. Doses of these agents, doses of tumor cells and lymphocytes and the results of the experiments are given in Tables 5 and 6.

The experiments indicate that conditioning immune animals with these agents did not reduce the humoral antibodies per unit volume of immune rat serum. Immune lymphocytes also retained their activity against the tumor regardless of treatment of the donor, as long as the lymphocytes remained alive. There was approximately the same reduction in the total quantities of humoral and cellular antibodies in the animals given $HN_2$ as in those treated with x-irradiation and cortisone, yet only the animals given the later treatment had a lowered resistance to the tumor. Therefore, an explanation of the difference between the effects of $HN_2$ and the combination of x-irradiation and cortisone on the immune mechanism was not apparent.

## Discussion

Nitrogen mustard and related cytotoxic drugs are now being tried as adjuncts to surgery for patients with cancer of internal organs in an effort to reduce local recurrences and to eliminate spread of cancer into the blood stream.[7, 16] This has stimulated efforts to define more completely the toxicity of these drugs as applied to patients undergoing operations. Morales and McDonald[6] showed that the toxicity of $HN_2$ is increased when administered to animals subjected to a standard type of abdominal operation. Other investigators have shown that these drugs cause a delay in the healing of experimental wounds.[3, 4]

The experiments reported in this paper were designed to investigate sublethal doses of alkylating cytotoxic drugs ($HN_2$ and TSPA) over a wide range and including doses suitable for patients undergoing surgery. These doses have no effect on the induced immunity of one strain of rats and the natural resistance of another strain to a transplantable rat lymphosarcoma. In contrast, combinations of x-irradiation and cortisone in doses which were equally well tolerated by the animals were shown to effect the host-tumor relationship to the disadvantage of the host in both strains of rats.

The assumption that animal experiments such as those we have described may be valid for the human being may lead to errors in interpretation. Animal experiments, however, are of great value in screening drugs for antitumor activity, and in determining the toxicity of drugs which are released for clinical trials. Our experiments indicate that the alkylating drugs now used as adjuncts to surgery, for regional perfusion and for wound irrigation have no effect on the cellular or humoral defenses which the host may possess against its neoplasm.

These experiments can also be interpreted to indicate that the cytotoxic drugs, $HN_2$ and TSPA, are less effective in conditioning animals to tissue transplants than are combinations of x-irradiation and cortisone at dose levels which permit the animals to remain in relatively good condition.

## Conclusions

Tumor immunity induced by growth and regression of a transplantable rat lymphosarcoma was broken down with x-irradiation and cortisone, but was not altered by $HN_2$ or TSPA.

The resistance of an inbred strain of rats to a homologous tumor transplant was lowered by x-irradiation and cortisone, but was not changed by $HN_2$ when these agents were given prior to the tumor inoculum.

Immune animals treated with $HN_2$ and with x-ray and cortisone had a diminished blood volume and a decrease of lymphoid tissue. Therefore, they probably had fewer humoral and cellular antibodies than untreated immune animals. However, only in those animals given x-ray and cortisone

was this decrease associated with an alteration of the immune response.

## Addendum

Since the preparation of our paper, work by McQuarrie and associates has come to our attention. These investigators showed that intensive treatment of rats and rabbits with nitrogen mustard in doses sufficient to produce and maintain marked leukopenia did not depress host response mechanisms to a degree sufficient to allow significant prolongation of homograft survival (McQuarrie *et al.:* Proc. Soc. Exp. Biol. and Med., **103**:278, 1960). These conclusions are in disagreement with those of Levinson and Necheles but substantiate our finding that $HN_2$ has no effect on tumor immunity. The explanation given by McQuarrie for the prolonged survival of grafts in the animals treated by Levinson and Necheles lies in their selection of experimental animals and in the absence of an adequate pairing between reciprocally grafted controls.

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### Discussion

PRESIDENT COLE: I realize that a presiding officer should not discuss many papers, but I would like to take advantage of Dr. Preston's kind invitation.

I think this is a very fine contribution containing some important basic data which should be valuable in the clinical aspects of this subject. As you know, there is considerable controversy regarding the effects of radiation and cortisone