INDUCTION OF TUMOR IN MICE WITH N,N-BIS(2-CHLOROETHYL)-N',O-PROPYLENEPHOSPHORIC ACID ESTER DIAMIDE (CYCLOPHOSPHAMIDE)*1

(Plate LXXVIII)

Shoji TOKUOKA

(Department of Pathology, Hiroshima University School of Medicine*2)

Synopsis

N,N-Bis(2-chloroethyl)-N',O-propylenephosphoric acid ester diamide (cyclophosphamide), one of the derivatives of nitrogen mustard, is said to be almost inactive in vitro, and the agent becomes biologically active in vivo only on appropriate activation, which is thought to be accomplished probably by the liver.

The present investigation deals with the late effect including possible carcinogenic influence of the agent in normal mice, to whom a minute amount of the agent was repeatedly administered for a relatively long duration.

The results obtained indicate that cyclophosphamide appears to be activated in vivo, probably by the liver, and to be capable of inducing benign and malignant tumors predominantly in the lung, liver, and sexual organs of both sexes in mice.

INTRODUCTION

The carcinogenicity of both sulfur and nitrogen mustards has been established experimentally by several investigators including Boyland et al.,4) Heston,9-12) and Griffin et al.7,8) In regard to carcinogenic effect of sulfur mustard in the human being, Yamada21,22) and Wada et al.19) reported a highly increased incidence of respiratory tract carcinoma among previous workers in a former war gas manufactory and called it an occupational Yperite gas cancer in the respiratory tract. Of the carcinogenicity of nitrogen mustard N-oxide, one of the derivatives of nitrogen mustards, experimental investigations have been performed by Warabioka20) and the author.15,16)

N,N-Bis(2-chloroethyl)-N',O-propylenephosphoric acid ester diamide (cyclophosphamide), a cyclic derivative of nitrogen mustard which was synthesized primarily by Arnold in 1957, has been known as one of "transport" form antitumor agents and was thought to be transformed into an "active" form in vivo through enzymatic hydrolysis by neoplastic cells.3) On the other hand, Tasaka et al.14) showed that the agent seems to be activated not only in neoplastic cells but also in certain normal tissue or organ. Further, Foley et al.6) pointed out that the agent is activated primarily by the liver.

*1 A part of this study was presented at 16th Meeting of Hiroshima Medical Association in 1964,17) and at 53rd Annual Meeting of Japan Pathological Society in 1964.18)

*2 Kasumi-cho, Hiroshima (德岡昭治).
The present paper deals with an investigation on whether the agent is not activated at all in normal tissue or organ, or is activated much or less in vivo and subsequently induces any late effect including possible carcinogenicity in normal mice.

**Materials and Methods**

Strains dd and A mice were used. They were given pelleted diet MF (Oriental Co., Tokyo) and water ad libitum, and the body weight of each mouse was measured every week throughout the experiments.

1. A total of 29 mice of dd strain of both sexes, 4 to 5 weeks old, were used. They were injected 5 mg/kg of cyclophosphamide saline solution (1 mg/ml solution) intraperitoneally twice a week for successive 15 weeks. As the control group, 20 mice of both sexes of the same strain were given 5 ml/kg of the normal saline solution intraperitoneally, twice a week for the same duration as the experimental group.

2. Mice of A strain, 25 of both sexes, 4 to 5 weeks old, were also given intraperitoneal injections of the same dose of cyclophosphamide saline solution, as in dd mice, twice a week for successive 15 weeks. For the control, 16 mice of both sexes of the same strain were given 10 ml/kg of a normal saline solution for the same duration.

All the mice were placed under observation until spontaneous death or until they were killed when dying, and were submitted to complete autopsy and histological examinations.

**Results**

In dd mice, changes of the mean body weight throughout the experiment was nearly the same between the experimental and control groups. On the other hand, in A mice, the mean body weight of each sex in the experimental group appeared to be slightly higher than that of the control group.

<table>
<thead>
<tr>
<th>Table I. Variety of Tumors Developed in dd and A Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>dd Mice</strong></td>
</tr>
<tr>
<td>Pulmonary adenoma</td>
</tr>
<tr>
<td>Pulmonary carcinoma</td>
</tr>
<tr>
<td>Liver cell adenoma</td>
</tr>
<tr>
<td>Testicular interstitial cell tumor</td>
</tr>
<tr>
<td>Mammary carcinoma</td>
</tr>
<tr>
<td>Leukemia</td>
</tr>
<tr>
<td>Miscellaneous</td>
</tr>
</tbody>
</table>

a) Adenomatous hyperplasia of bronchial epithelium  
b) Testicular interstitial cell hyperplasia  
c) One each of fibrosarcoma of mammary gland region, liposarcoma on back, and uterine leiomyoma  
d) Adenomatous goiter of thyroid gland  
e) Orbital papillary adenoma
In dd mice 12 (55%), of 22 mice which survived beyond 48 weeks following the beginning of the treatment developed tumors in various organs and in the control group, 3 (30%) of 10 mice which survived beyond the same period developed tumors.

In A strain, 6 (37.5%) of 16 mice which survived beyond 42 weeks following the beginning of the experiment developed tumors, while in the control group, 2 (18.1%) of 11 mice developed tumors.

In dd mice, tumors developed preponderantly in the lung, liver, testis, and mammary gland as shown in Table I. There were a few mice which had more than two different tumors in different organs simultaneously. On the other hand, in A mice almost all tumors were seen only in the lung. Besides tumors, there were a few cases that revealed interstitial cell hyperplasia of the testis, as well as adenomatous hyperplasia of bronchial epithelium in the lung and adenomatous goiter of the thyroid gland, in experimental groups of both strains. Degenerative changes were frequently observed in liver cells and tubular epithelium of the kidney in experimental group of both strains.

**Discussion**

The tumor incidence in both strains appeared higher in the experimental group than that of the control group (dd strain, \( p = 0.183 \); A strain, \( p = 0.263 \), by Fisher's exact method). Similar observation has recently been reported by Duhig,\(^5\) in which strain A mice were given intraperitoneal administration of 0.02 mg of cyclophosphamide saline solution once a week for successive 5 weeks and the incidence of pulmonary adenoma was higher than that of untreated mice. Therefore, it seems almost reasonable to say that the agent was apparently activated *in vivo* and ultimately induced the tumor.

Tumors were seen more in various organs in mice of dd strain than in A strain as shown in Table I. Particularly in dd male mice, 7 out of 10 developed tumors (70%), and 4 of these 7 mice had interstitial cell tumor of the testis, and another 2 of them had liver cell adenoma. There was a mouse which developed both interstitial cell tumor of the testis and liver cell adenoma simultaneously. Moreover, 2 of these 4 testicular tumor cases had simultaneous adenomatous goiter of the thyroid gland. A case with bronchogenic adenocarcinoma of the lung revealed widespread metastasis to the regional lymph node, heart, and bilateral kidneys.

In female mice of dd strain, on the other hand, there was no apparent evidence of tumor development in the ovary, although tumors were frequently seen in their mammary gland and uterus. Two of 3 cases with adenocarcinoma of the mammary gland revealed multiple metastases to the lung.

In summary, in both male and female mice, it was noted particularly in dd mice that neoplastic changes were predominantly seen in the sexual organs, as well as in the lung and the liver, and many of the tumors that developed in these experimental mice were more malignant than those of the control mice. In addition, experimental induction of interstitial cell tumor of the testis seemed rather rare and only a few re-
ports dealing with testicular interstitial cell tumors induced by either estrogen or cadmium have been reported by several investigators.\(^1\),\(^2\),\(^3\)

On the bases of these facts that the degenerative and neoplastic changes were seen not infrequently in the liver and that the development of tumors was more often seen in the sexual organs, it was thought that the agent was most likely activated by the liver in these mice, as Foley et al. have postulated, and induced secondary endocrine abnormalities somewhat resembling that of estrogen hyperactivity.

It is of interest to note that the rôle of induction of tumors in mice following cyclophosphamide administration might be related to endocrine abnormalities secondary to liver damage, although possible direct influence of the agent on sexual organs through the liver is undeniable at the present time. Further investigation particularly from this view is now in progress.

The author wishes to express his gratitude to Professor Dr. A. Yamada of this University for his valuable advice and encouragement throughout the present study. This work was supported in part by Public Health Service Research Grant OH-00189 from the Division of Occupational Health, National Institutes of Health, U.S.A.

(Received June 7, 1965)

References

EXPLANATION OF PLATE LXXVIII

Photo 1. Interstitial cell tumor of the testis (H-E stain), in a mouse of dd strain, given 5 mg/kg of cyclophosphamide twice a week for successive 15 weeks and died on 509th experimental day.

Photo 2. Same as Photo 1. Higher magnification of testicular tumor (H-E stain).

Photo 3. Bronchogenic adenocarcinoma of the right lung (H-E stain) in a male mouse of dd strain treated with cyclophosphamide and died on 387th experimental day. The tumor had widespread metastasis to the lymph node, heart, and bilateral kidneys.

Photo 4. Liver cell adenoma (H-E stain) in a male mouse of dd strain treated with cyclophosphamide and died on 526th experimental day. The tumor cells revealed moderate atypicality.

H-E=Hematoxylin and Eosin stain