Studies on the Mechanism of Action of Cytoxan  
Evidence of Activation in Vivo and in Vitro*  
G. E. FOLEY, O. M. FRIEDMAN, AND B. P. DROLET  
(Laboratories of Microbiology, Children's Cancer Research Foundation, and Department of Pathology,  
Harvard Medical School, Children's Hospital Medical Center, Boston, Massachusetts;  
and Department of Chemistry, Brandeis University, Waltham, Mass.)  

SUMMARY  
The reported inactivity of Cytoxan, when assayed directly in mammalian cell cultures, has been confirmed, even with cell lines derived from experimental tumors which respond in vivo to Cytoxan therapy.  
However, blood serum or crude liver extracts from rats treated with Cytoxan were inhibitory in vitro for serially propagated mammalian cells derived from normal or neoplastic tissue, as well as for cells in primary culture.  
Experiments in which Cytoxan was incubated in vitro in homogenates of normal and neoplastic mouse tissue indicate that, of the tissues so examined under the present experimental conditions, only the liver homogenates contained a substance (or substances) inhibitory for mammalian cells in culture.  
The experimental data presented herein support the hypothesis that Cytoxan is a “transport” structure which becomes biologically active only upon appropriate “activation,” and that “activation” sufficient to interfere with cell growth, as adjudged by the present in vitro methods of assay, is accomplished primarily (but probably not exclusively) by the liver.

Cytoxan1 (2H-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl) amino]tetrahydro-2-oxide), synthesized by Arnold, Bourseaux, and Brock (1, 3), is closely related to a series of N-phosphorylated derivatives of bis-β-(chloroethyl)amines (Chart 1) prepared originally by Friedman and Seligman (17) as models for potentially enzyme-activated antitumor agents following the observation that nitrogen mustards are essentially detoxified by N-phosphorylation (25). The structure of Cytoxan differs by the insertion of a 3-carbon bridge between the hydroxy and amino groups of N,N-di-(2-chloroethyl)phosphorodiamidic acid, which was reported by Friedman and Seligman in 1954 (17). This same publication also reported the synthesis of a compound, phenyl N,N-di-(2-chloroethyl) phosphorodiamidate (17), in which a phenyl ring was attached to the oxygen on phosphorus (Chart 1). Although N,N-di-(2-chloroethyl) phosphorodiamidic acid was unstable at pH 5.0, evidence of enzymatic hydrolysis was obtained in mammalian tissue homogenates. The phenyl-substituted derivative was not hydrolyzed spontaneously or enzymatically, however, under the same experimental conditions.3  
According to the mechanism postulated, N-phosphorylated derivatives of bis-β-(chloroethyl) amine (16, 17, 26, 27), including Cytoxan (1, 3, 4), represent “transport” forms which presumably must be transformed to “active” structures in vitro. In the case of Cytoxan, Arnold et al. (1, 3, 4)
**N,N-Di-(2-chloroethyl)-phosphorodiamic acid** (Friedman and Seligman, 1954)

**2H-1,3,2-oxazaphosphorine, 2-oxide** (2-chloroethyl)aminotetrahydro-2-oxide (Arnold, Bourseux and Brock, 1958)

**Phenyl-N,N-di-(2-chloroethyl)-phosphorodiamic acid** (Friedman and Seligman, 1954)

**Chart 1.**—Structural relationship of Cytoxan to previously synthesized derivatives of N-phosphorylated bis-β-chloroethylamines (Friedman and Seligman, cf. ref. 17).

Further postulated that the "active" derivative is an open-chain structure resulting from enzymatic hydrolysis by neoplastic cells (Chart 2), although there is no evidence for the presence of the necessary enzymes in neoplastic cells, and, indeed, Brock (4) reported Cytoxan to be stable when incubated in vitro in suspensions of Yoshida sarcoma cells.

Cytoxan has been reported to be inactive in mammalian cell cultures (5), although exhibiting an interesting degree of antitumor activity both in experimental animals and man (2-6, 20, 21, 28, 29). This lack of inhibitory activity for mammalian cells in vitro has been confirmed, even with cell lines derived from experimental tumors (e.g., Sarcoma 180 and L1210 leukemia) which are inhibited by Cytoxan in vivo (5, 28, 29).

If the activity of Cytoxan and other N-phosphorylated derivatives of bis-β-(chloroethyl)amine is dependent upon hydrolysis at one or more of the loci indicated in Chart 3, it would appear that appropriate activation is accomplished in vivo but not under the experimental conditions of mammalian cell culture. This inactivity in vitro might be merely a reflection of the loss of the enzyme(s) essential for activation by mammalian cells consequent to serial propagation in culture; or, alternately, Cytoxan may undergo hydrolysis in vivo...
at loci other than neoplastic tissue, producing either directly or via intermediary structures an inhibitory end product. The present studies were undertaken to obtain evidence for the "activation" of Cytoxan in biological systems in support of either of these hypotheses. The chemical and enzymatic considerations pertinent to the identification of the biologically active derivatives of Cytoxan will be presented elsewhere.3

MATERIALS AND METHODS

Mammalian cell culture.—The isolation and serial propagation of the CCRF cell lines (CCRF-180, from Sarcoma 180 in CFW mice [12, 13]; CCRF-1210, from L1210 leukemia in DBA/2 mice; CCRF-1534, from P-1534 leukemia in DBA/2 mice [13, 14]; CCRF-91, from Cloudman melanoma in DBA/1 mice [13, 14]; CCRF-109, from DBRB mammary adenocarcinoma of DBA/1 mice; and CCRF-217, from testes, normal CFW mice) have been described in detail elsewhere(13). All assays were done in Eagle's basal medium (9), supplemented with 10 per cent whole calf serum (13). The methods of mammalian cell culture assay, described in detail elsewhere (10, 11, 15), were as follows: inocula of 200,000 cells from actively proliferating stock cultures were distributed in 2.5-ml. aliquots of basal medium and allowed to attach to the surface of stationary culture vessels overnight at 37° C. The protein content of the inoculum (base-line values) was determined on a series of culture vessels, according to the method described by Oyama and Eagle (23), and the medium in the assay cultures was then replaced with 2.5 ml. of basal medium containing the desired concentration of the test sample to be assayed. The fluid portion of cultures containing such test samples was renewed at three successive 24-hr. intervals, and final protein content was determined after the 5th day at 37° C. Each series of experiments was done in duplicate or triplicate.

Primary cell cultures.—Tumor specimens were treated as previously described (13, 15), and the resulting cell suspensions were distributed in cul-
ture flasks at ca. 400,000 cells in 2.5 ml. of basal medium. After the cells had become attached to the glass, cultures of comparable density were selected by microscopic inspection, base-line protein content determined, and the remaining cultures used without serial passage for mammalian cell assays, as described above.

In vivo samples.—Cytoxan was administered intraperitoneally to Fischer or Sprague-Dawley rats, 170–200 gm. wt., as single doses of 0.75 or 1.0 gm/kg, or as four doses of 0.5 gm/kg at approximately 10-hour intervals. Rats pooled by dose were sacrificed at varying intervals post-treatment, and blood serum was collected by exsanguination. Spleens, kidneys, and livers were excised, mechanically homogenized, and extracted (separately) as 10 per cent or 20 per cent suspensions by weight in Krebs-Ringer phosphate buffer (7) or Eagle’s basal medium. The homogenates were clarified by centrifugation, filtered through moist "Filter Cel" and sterilized by filtration through a millipore membrane. Normal, untreated rats of similar weight and sex were treated as above to provide control sera and tissue extracts. Extracts of mouse neoplastic and normal tissue were similarly prepared. All specimens were frozen at —15° C. until assayed.

In vitro samples.—Cytoxan (1.0 or .50 mg/ml) was incubated in air at 37° C. with gentle shaking in 5.0 ml of: (a) normal, pooled rat serum, (b) pooled defibrinated rat blood, or (e) homogenates (or slices) of mouse and rat tissue (0.5 or 1.0 gm. of tissue per 5.0 ml. Krebs-Ringer buffer or Eagle’s basal medium) in a Dubnoff metabolic water bath for periods of 1 or 2 hr. The "buffy coat" containing the tumor cells from the ascites form of L1210 leukemia in DBA/2 mice was collected and separated as described previously (13), "packed" in graduated centrifuge tubes at 50–70 X g for 2–3 min., and 0.5 or 1.0 ml. of "packed" cells were suspended in 5.0 ml of Krebs-Ringer or basal medium and incubated with Cytoxan as above. Clarification and sterilization when indicated were accomplished in the same manner as that described for the preparation of the tissue extracts from Cytoxan-treated rats.

**TABLE 1**

<table>
<thead>
<tr>
<th>Basal Medium + Cytoxan†</th>
<th>Normal Rat Serum</th>
<th>Normal Rat Serum Incubated with Cytoxan (1 mg/ml, in Vitro)‡</th>
<th>Serum from Rats Treated with Cytoxan (500 mg/kg, I.P.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal Medium</strong></td>
<td><strong>None</strong></td>
<td><strong>Post-injection 10%</strong></td>
<td><strong>10%</strong></td>
</tr>
<tr>
<td>Normal Rat Serum</td>
<td>7.8</td>
<td>8.0</td>
<td>9.0</td>
</tr>
</tbody>
</table>

* Similar results obtained with CCRF-1584.
† Similar results obtained with all cell lines so studied. Cf. text.
‡ Similar results obtained with normal, defibrinated rat blood.

RESULTS

**Rat sera.**—Serum samples collected as soon as 15 and 30 minutes following a single intraperitoneal dose of 500 mg/kg of Cytoxan exhibited marked inhibitory activity in mammalian cell cultures, as illustrated by the typical experiments with CCRF-1210 summarized in Table 1. It is evident that the sera of Cytoxan-treated rats contained a substance which inhibits cell growth; a concentration of as little as 2.0 per cent treated serum resulting in ca. 50 per cent inhibition of protein synthesis. That this substance was not present in normal, untreated rat sera was emphasized not only by the lack of inhibitory activity in such sera but by their stimulatory effect on cell growth as well, as indicated by the increased protein content of control cultures containing pooled, normal rat serum (Table 1). Similarly, there was no evidence of inhibitory activity in normal rat serum or normal, defibrinated rat blood in which Cytoxan had been incubated *in vitro* (Table 1).
The inhibitory activity of Cytoxan-treated rat sera was in sharp contrast to the inactivity of Cytoxan, even in concentrations of 1.0 mg/ml, when assayed directly in the same mammalian cell systems (Table 1). Cytoxan was equally inactive in similar experiments with serially propagated cell lines derived from S-180, P-1534 leukemia, S-91 melanoma, DBRB mammary adenocarcinoma, a human epidermoid carcinoma (8), normal mouse testes, normal fibroblasts derived from the Chinese hamster;6 and with primary cultures derived from mouse tissue (e.g., S-180) to which Cytoxan had been added before any appreciable degree of cellular multiplication had occurred.

Tissue extracts.—The results obtained with assays of tissue extracts prepared from Cytoxan-treated and normal rats are illustrated in Table 2. Extracts of liver exhibited marked inhibitory activity, in contrast to the trace of activity in kidney extracts, and the lack of such activity in spleen extracts. The assay of such liver extracts as illustrated by the inhibitory activity of control, normal rat liver (Table 2). However, despite this complicating toxicity, there was a pronounced difference in the inhibitory activity of extracts of normal and Cytoxan-treated rat liver (Table 2).

The inhibitory activity of sera and tissue extracts prepared from Cytoxan-treated rats disappeared within relatively short periods of time, as illustrated by the experiments summarized in Chart 4. The inhibitory activity exhibited by concentrations of 5.0 per cent and 2.0 per cent treated serum at 15 and 30 min., and 1 and 2 hr. following a single intraperitoneal dose of 0.75 gm/kg of Cytoxan, had disappeared by 4 hr., as indicated by comparison of the protein content of treated and control cultures. With serum samples collected 4 hr. following administration of Cytoxan, ca. 10 per cent treated serum was required for 50 per cent inhibition of protein synthesis, and inhibitory activity had disappeared completely, even from 10 per cent concentrations of serum collected 24 hr. following administration of Cytoxan.

Tissue homogenates.—The results obtained with Cytoxan incubated in vitro in the presence of homogenates of neoplastic and normal mouse tissue are illustrated in Table 3. There was no evidence of inhibitory activity in the supernatants of the homogenates of neoplastic tissue. Experiments with homogenates of normal tissue again were complicated by their inherent protein content and toxicity for mammalian cells in culture, as

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (per cent)</th>
<th>Increase in protein content (referred to base-line as 1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Spleen</td>
</tr>
<tr>
<td>Cytoxan,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 mg/kg</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>4X q 10 hr.</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.6†</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>2.2†</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.7</td>
</tr>
<tr>
<td>Control, basal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>medium</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Similar results obtained with CCRF-1584.
† Cells remaining on glass exhibited same morphologic changes induced by exposure to Cytoxan-treated rats.
‡ Inhibitory activity enhanced by heating 5 min. at 60°-65°C.

by the present methods was complicated by their inherent protein content and toxicity for mammalian cells in culture, as has been observed by others in similar mammalian cell systems7 and

6 Obtained through the courtesy of Dr. George Yerganian, Laboratory of Cytogenetics, The Children's Cancer Research Foundation.

7 Personal communications, Dr. C. G. Smith, The Upjohn Company, Kalamazoo, Mich.
evidenced by the inhibitory activity exhibited by the control homogenates (Table 3). The liver homogenates incubated with Cytoxan, however, exhibited increased inhibitory activity as compared with the control liver homogenates or the homogenates of spleen and kidney incubated with or without Cytoxan (Table 3). This difference became even more evident with direct microscopic examination of the assay cultures, as illustrated in Figures 1 and 2.

**Morphology.**—In addition to the marked inhibition of protein synthesis, sera or liver extracts from Cytoxan-treated rats, and homogenates of mouse liver incubated with Cytoxan in *vitro* induced pronounced morphologic aberrations in partially inhibited assay cultures. As illustrated in Figures 3 and 4, such cultures consisted largely of multinucleated giant cells with scanty cytoplasm, suggesting metaphase arrest and endoreduplication. These morphologic changes in some respects resembled those observed in *vivo* with ethylenephosphoramides (24) and nitrogen mustard (18). Similar morphologic aberrations also have been observed with cultures of normal embryonal fibroblasts derived from the Chinese hamster (*Cricetulus griseus*) exposed to Cytoxan-treated rat sera. These morphologic changes have not been observed following *in vitro* exposure to Cytoxan itself, to Cytoxan incubated *in vitro* in normal rat sera or defibrinated rat blood, or to normal rat sera alone.

**DISCUSSION**

The experimental data presented herein support the hypothesis that Cytoxan is indeed a “transport” structure, which becomes biologically active only upon appropriate “activation.” The lack of inhibitory activity in normal rat serum and normal, defibrinated rat blood incubated with Cytoxan *in vitro*, as contrasted with the inhibitory activity of the sera of rats treated *in vivo* with Cytoxan, suggests that activation does not occur to any appreciable extent in rat blood per se. Similarly, the inability of homogenates of neoplastic tissue to effect this “activation” suggests that the essential structural alteration probably is not accomplished in neoplastic tissue per se to a sufficient extent to interfere with cell growth, at least by those mouse tumors examined herein. Inhibitory activity, on the other hand, can be demonstrated in the blood serum, in extracts prepared from the livers, and to a lesser extent, the kidneys of rats treated with Cytoxan *in vivo*; and in the homogenates of mouse liver incubated *in vitro* with Cytoxan. Although the present experiments with relatively crude extracts and homogenates of liver are complicated by the inherent protein content and toxicity of such preparations for mammalian cells in *vitro*, there is reasonable evidence for the conclusion that Cytoxan is activated primarily (but probably not exclusively) by the liver.

The relatively rapid disappearance of inhibitory activity from the concentrations of blood serum and tissue extracts of Cytoxan-treated rats assayed in mammalian cell cultures herein probably bears no relationship to the reported effectiveness of intermittent Cytoxan therapy *in vivo* (21, 28), since it is probable that inhibitory activity persists *in vivo* at effective levels which are beyond the limits of the sensitivity of the present methods of assay when applied to whole serum or crude tissue extracts.

The evidence accumulated from these and other studies (5) indicates thus far that serially propagated mammalian cell lines of either normal or neoplastic origin do not activate Cytoxan *in vitro*, as indicated by the lack of inhibitory activity of Cytoxan when assayed directly with such cell lines. The similar inability of mammalian cells in primary culture and homogenates of certain

---

**TABLE 3**

<table>
<thead>
<tr>
<th>Source of Homogenate*</th>
<th>Increase in Protein Content (Referring to Normal as 1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubated 6 hr., 37°C, with Cytoxan (5.0 mg/ml)</td>
</tr>
<tr>
<td>Neoplastic:</td>
<td></td>
</tr>
<tr>
<td>P-1534</td>
<td>5.6</td>
</tr>
<tr>
<td>L1210†</td>
<td>6.2</td>
</tr>
<tr>
<td>S-180</td>
<td>5.3</td>
</tr>
<tr>
<td>S-91</td>
<td>5.2</td>
</tr>
<tr>
<td>DBRB</td>
<td>5.3</td>
</tr>
<tr>
<td>Normal:</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1.8</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.8</td>
</tr>
<tr>
<td>Control, basal medium</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* Tested as 10% and 20% suspensions in Krebs-Ringer. Supernates assayed at 15% final concentration in cell cultures. Essentially similar results obtained in replicate experiments with aliquots of homogenates incubated in basal medium.
† Prepared from packed cells from ascites form in DBA/2 mice.

---

These studies were done by Dr. George Yerganian, Laboratory of Cytogenetics, Children's Cancer Research Foundation. Detailed cytological studies are now in progress.
Fig. 1.—Effect of homogenate of normal mouse liver incubated with Cytoxan on growth of CCRF-1210 cells in vitro (cf. Table 3).

Fig. 2.—Aliquot of same homogenate incubated without Cytoxan. Five-day assay cultures, unstained cells in situ, X210.

Fig. 3.—Effect of sera of Cytoxan-treated rats on morphology of CCRF-1210 cells in vitro. Five per cent rat serum collected 30 min. following 0.5 gm/kg Cytoxan, intraperitoneally (cf. Chart 1). Partially inhibitory concentrations of extracts of liver of Cytoxan-treated rats, or homogenates of normal mouse liver incubated with Cytoxan in vitro induced similar morphologic changes.

Fig. 4.—10 per cent normal rat serum controls. Five-day assay cultures, celloidin fixation in situ (22), H & E, X340.
freshly excised normal and neoplastic tissue to activate Cytoxan suggests that this failure may not be due exclusively to loss of physiologic function consequent to serial propagation in vitro, but may well be the result of the inherent inability of the tissues from which the cell lines were derived to accomplish appropriate activation, viz., a cell line derived from the physiologically active structures of the liver (or kidney) might effectively activate Cytoxan in vitro. An alternate possibility is that the preliminary alteration essential to activation occurs at a site in the host other than in the tumor, as has been suggested previously for certain other antitumor agents which are inactive in mammalian cell culture (11,19), resulting in an intermediary structure, which in turn is further modified by neoplastic (and other) cells in vitro and in vivo, with the consequent release of a biologically active structure. Experiments designed to further examine these points are now in progress.

REFERENCES
Studies on the Mechanism of Action of Cytoxan Evidence of Activation \textit{in Vivo} and \textit{in Vitro}

G. E. Foley, O. M. Friedman and B. P. Drolet