Synergistic Antileukemic Effect of Theophylline and 1,3-Bis(2-chloroethyl)-1-nitrosourea

William D. DeWys and Sudha H. Bathina

ABSTRACT

A series of experiments evaluated the antileukemic effect of an agent which elevates cellular cyclic adenosine 3':5'-monophosphate levels by inhibiting phosphodiesterase. When administered alone, theophylline had only modest antileukemic effects, but it had synergistic effects when administered with 1,3-bis(2-chloroethyl)-1-nitrosourea. This synergy produced an improved therapeutic index in a dose-response study and in a comparison between antileukemic effects and effects on white blood cell nadirs. Uptake of 1,3-bis(2-chloroethyl)-1-nitrosourea and studies of timing of treatment support the hypothesis that elevation of cyclic adenosine 3':5'-monophosphate levels is the mechanism of the observed synergistic effect.

INTRODUCTION

cAMP is known to play an important role in controlling cell growth (13). Caffeine and theophylline, which inhibit cAMP phosphodiesterase and thus raise cAMP levels, slowed the growth of normal and transformed hamster cells (2). cAMP and its analogs act to inhibit growth of a spectrum of normal and transformed cells (14, 15).

In vitro treatments which are known to increase cAMP also inhibit DNA synthesis in chronic lymphocytic leukemia cells (9). These and other in vitro studies have led to studies on the effects of cAMP on growth of tumor cells in vivo. Administration of cAMP, dbcAMP, or other agents known to elevate cAMP levels have slowed the growth of Ehrlich tumor cells (16), lymphosarcoma (8), mammary tumors (4), and Walker 256 carcinoma (3).

However, no response to dbcAMP was seen in leukemia L1210, a tumor which is widely used to select treatments for possible clinical application. Elevation of cAMP levels would represent an approach to clinical anticancer treatment different from those approaches currently in use and thus possibly be of value in combination with existing therapies. We therefore evaluated an agent that elevates cAMP levels for antileukemic effect singly and in combination with the cytotoxic anticancer drug, BCNU. BCNU was selected based on its effectiveness in leukemia L1210.

MATERIALS AND METHODS

Mice. Male C57BL x DBA/2 mice weighing 18 to 25 g (The Jackson Laboratory, Bar Harbor, Maine) were housed in groups of 10 in plastic cages and fed Purina laboratory chow ad libitum.

Tumor. Leukemia L1210 (10⁶ cells/mouse) was transplanted i.p. on Day 0 as described previously (7). Survival of mice was recorded daily for 30 days. Mice remaining after this period of observation were considered cured, since one viable leukemia cell would cause death in approximately 15 days (17).

Drugs. Theophylline (1,3-dimethylxanthine) was purchased from Sigma Chemical Co., St. Louis, Mo., and was administered as an aqueous solution (pH 7 to 8). BCNU (NSC 409982), supplied by the Division of Cancer Treatment, National Cancer Institute, NIH, was prepared immediately prior to use by dissolving it first in ethanol and then diluting it with sterile water to the desired concentration. [2-¹⁴C]BCNU (Lot 2153-112) was also obtained from the Division of Cancer Treatment, National Cancer Institute, NIH. All drugs were administered i.p. in a dose of 0.01 ml/g body weight. BCNU was given on Day 1, 24 hr after the tumor implant. Treatment schedules and dosages are given in the tables and legends of the charts. Unless otherwise specified, when theophylline was given in combination with BCNU, theophylline was given first, followed within 15 min by BCNU.

Tumor Measurement. Ascites leukemia cells in control and theophylline-treated mice were collected by serial washing of the peritoneal cavity (7, 10). Acetic acid was used to lyse erythrocytes, and counts were made on a hemocytometer. Three mice were used for each data point.

Assay for cAMP. Tumor cell extracts for the assay of cAMP were prepared according to the method of Coe (5). Tumor-bearing mice were sacrificed by cervical dislocation on Day 5 at timed intervals after a single dose of theophylline; ascites was quickly drawn and blown into 7% ice-cold perchloric acid. Potassium bicarbonate (30%) was used to neutralize the extract, and the resulting potassium perchlorate was removed by centrifugation after allowing it to stand in an ice bath for 2 hr. The supernatants were used directly for determining the cAMP levels. The protocol for the assay was a modification of the procedure of Tovey et al. (20) (Diagnostic Products Corp., Los Angeles, Calif.). This method assays a cAMP concentration range of 0.11 to 27 pmol/tube for the standard curve. Tumor from at least 3 mice was used for each time point. cAMP levels are expressed as pmol/10⁶ cells.

Cellular Uptake of BCNU. Transport studies were performed on suspension cultures of L1210 cells using a modification of the method of Begleiter (1). L1210 cells were suspended in Roswell Park Memorial Institute Tissue Culture Medium 1640.
media of a concentration of $4 \times 10^6$ cells/ml. Theophylline was dissolved in distilled water (pH 7.0) and added to the reaction mixture at a final concentration of 3 mg/ml. [\textsuperscript{2-\textsuperscript{14}C}] BCNU was diluted with cold BCNU to a concentration of 0.4 mg/ml and a radioactivity of 1 $\mu$Ci/ml in the reaction mixture. Incubation tubes were set up in duplicate with or without theophylline in an oscillating water bath at 37°. The incubation was timed beginning with the addition of BCNU. The reaction was stopped by adding 10 volumes of iced media. Cells were centrifuged with refrigeration and washed once with iced media. The cells were then lysed with distilled water, solubilized with 0.5 N NaOH, and counted in aquosol. Begleiter et al. (1) have documented that intact BCNU in the cells is measured in this assay.

WBC. For peripheral WBC, blood was obtained from orbital sinus and counted using a hemocytometer after appropriate dilutions with acetic acid and expressed as WBC per ml.

Statistical Analysis. When the number of mice surviving treatment was greater than zero but less than the total group, the expected number of surviving leukemic cells per mouse in the whole group was determined by application of the Poisson distribution (17).

RESULTS

Antileukemic Effect of Theophylline and BCNU. The effect on survival for treatment with theophylline alone, BCNU alone, or their combination is shown in Table 1. Theophylline alone increased median survival by 1 day. When theophylline was given with BCNU, it increased the median survival 3 to 6 days and strikingly increased the number of 30-day survivors compared to that with BCNU alone. Aminophylline (another potent inhibitor of phosphodiesterase) and dbcAMP, when given alone or in combination with BCNU, produced results similar to those with theophylline in terms of the median and long-term survival of mice (data not shown).

The effect of the combination of theophylline and BCNU may be interpreted by application of the Poisson distribution to the number of mice surviving as described by Skipper (17). When the number of survivors per group is greater than zero but less than the total, one can estimate the expected number of surviving leukemic cells per mouse for the group (Table 1, Column 5). This analysis leads to the conclusion that the addition of theophylline results in killing of 83 to 92% of the cells that would have survived BCNU alone. Since the 1-day increase in survival for theophylline alone is consistent with a 75% reduction in cell survival (based on a cell-doubling time of 12 hr), this analysis based on the Poisson distribution suggests a greater antileukemic effect attributable to theophylline given with BCNU than for theophylline given alone.

To evaluate this possibility further, we measured the antileukemic effect by direct cell counts. Serial ascites cell counts after theophylline administered 3 times daily for 2 days beginning on the third day after L1210 implant are shown in Chart 1. By 24 hr after initiation of treatment, there was a drop in the cell count to a level that was 19% of the concurrent untreated control value. Between 24 and 48 hr of treatment, there was a 2.8-fold increase in cell count in spite of continued theophylline treatment. This multiple-dose study established that the nadir leukemic cell count occurred at 24 hr, and this time point was used in subsequent single-dose studies.

The effect of graded single doses of theophylline on cAMP levels in L1210 cells and on leukemia cell kill after theophylline alone or theophylline plus BCNU is shown in Table 2. cAMP levels 1 hr after theophylline (see below for the basis of the 1-hr interval) increased progressively with the increasing dose of theophylline. The percentage reduction in survival of leukemia cells (at 24 hr) also increased with the increasing dose of theophylline. The percentage reduction in survival of leukemia cells was thus proportionate to the degree of elevation of the level of cAMP. When theophylline was given with BCNU, a dose-related increase in the percentage of cell kill attributable to theophylline was also seen. The percentage of cell kill attributable to theophylline given with BCNU was greater than

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Median survival time (days)</th>
<th>30-day survivors</th>
<th>Expected no. of surviving leukemic cells/mouse</th>
<th>% of theophylline-caused cell kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>7 (7 - 8)</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Theophylline</td>
<td>8 (7 - 11)</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCNU</td>
<td>15 (14 - 20)</td>
<td>2/10</td>
<td>1.6</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>BCNU + theophylline</td>
<td>17 (18)</td>
<td>7/8 (9)</td>
<td>0.12</td>
<td>92</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>8 (7 - 8)</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCNU</td>
<td>13 (12 - 14)</td>
<td>1/10</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BCNU + theophylline</td>
<td>19 (14 - 20)</td>
<td>7/10</td>
<td>0.4</td>
<td>83</td>
</tr>
</tbody>
</table>

* Initiated i.p. on Day 1. Theophylline (150 mg/kg) was given twice daily on Days 1 to 4; BCNU (20 mg/kg) was given as a single dose on Day 1.
* Ten mice in each group.
* Based on Poisson distribution as described by Skipper et al. (17).
* Obtained for each experiment by converting data in Column 4 as follows:

\[
\% \text{ of cell kill for theophylline} = 100 \times \frac{\text{no. of cells/mouse for BCNU + theophylline}}{\text{no. of cells/mouse for BCNU alone}}
\]

* Number in parentheses, range.
* Median not shown, only one death in group.
* Only 6 evaluable in this group because of 2 accidental deaths due to i.p. injection of drug.
Theophylline alone or with BCNU produced a significant lowering of WBC compared to control. The effect of theophylline on WBC was dose-dependent, with higher doses resulting in greater suppression of WBC. The addition of theophylline to BCNU further enhanced the effect, as evidenced by a more pronounced drop in WBC compared to BCNU alone.

**Analysis of Synergism.** The concept of synergism was tested by comparing the effect of theophylline added to BCNU on killing of normal hematologic cells and on leukemic cells. The effect of BCNU and theophylline on peripheral WBC 4 days after treatment is shown in Table 2. Based on a cell-doubling time for leukemia L1210 of about 12 hr, a 1-day increase in median survival represents one-fourth as many cells surviving treatment. The results for a BCNU dose of 5.8 mg/kg (Table 4) are a 1.5-day increase in median survival for the addition of theophylline compared to BCNU alone, consistent with a ratio of surviving leukemia cells of 0.12. For the BCNU dose of 8.8 mg/kg, a 4-day increase in median survival is interpreted as a surviving ratio of 0.004 for BCNU plus theophylline versus BCNU alone. For the higher doses of BCNU, greater than 50% of the mice receiving theophylline plus BCNU survived, precluding this type of analysis. These 2 studies (Table 3 and 4) lead to the conclusion that the cytotoxic effect of BCNU for normal cells was increased less than 2-fold, whereas the killing of leukemic cells was increased as much as 8-fold or more by the addition of theophylline.

Higher doses of BCNU were studied in another experiment, and the nadir (Day 4) of WBC is displayed in relationship to the percentage of 30-day survivors (Chart 2). Comparing equally effective doses, lower nadir counts were observed with BCNU than with the combination of BCNU and theophylline (compare 30 mg BCNU and 10 mg BCNU plus theophylline). Also at equitoxic doses, a larger number of 30-day survivors were noted with BCNU plus theophylline than with BCNU alone (compare 20 mg BCNU and 10 mg BCNU plus theophylline).

Since blood count results represent only one aspect of toxicity, a full range of doses was studied, using survival as a more general measure of toxicity (Chart 3). The right-hand side of the dose-response curve (toxicity) was comparable in normal and leukemic mice. Overall, the dose-response curve for BCNU alone was more narrow than that for BCNU plus theophylline. BCNU alone gave 90% survivors at a dose of 40 mg/kg, but the next higher dose tested was toxic, as seen by the sharp drop in the survival percentage in normal and leukemic mice. BCNU given with theophylline resulted in 60% survival at 60% survival at a dose of 10 mg/kg, 100% survival at 20 mg/kg, and 30% survival at 30 mg/kg, indicating a broad therapeutic range.

The BCNU dose estimated by interpolation to cause 50% drug-related mortality in leukemic mice was 44 mg/kg for BCNU alone and 27 mg/kg for BCNU when given with theophylline. The dose of BCNU estimated to be effective in eradicating leukemia in 50% of the treated mice, interpolated from the left side of the dose-response curve in Chart 3, was 33 mg/kg for BCNU alone and 8 mg/kg for BCNU when given
Table 4

Effect of BCNU and theophylline on mouse survival

Mice were given $10^4$ L1210 cells i.p. on Day 0 and single doses of BCNU or BCNU plus theophylline (150 mg/kg) on Day 1 and then were followed for survival.

<table>
<thead>
<tr>
<th>BCNU (mg/kg)</th>
<th>Theophylline + BCNU</th>
<th>BCNU alone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median survival</td>
<td>Median survival</td>
</tr>
<tr>
<td></td>
<td>time (days)</td>
<td>time (days)</td>
</tr>
<tr>
<td>5.8</td>
<td>12 (10-13)</td>
<td>10.5 (9-11)</td>
</tr>
<tr>
<td>8.8</td>
<td>15 (11-15)</td>
<td>11 (10-12)</td>
</tr>
<tr>
<td>13.3</td>
<td>(11-18)</td>
<td>12 (11-16)</td>
</tr>
<tr>
<td>20.0</td>
<td>100 (15-18)</td>
<td>15 (15-18)</td>
</tr>
</tbody>
</table>

* Since leukemia L1210 cells double about every 12 hr, a 1-day increase in median survival represents one-fourth as many cells surviving treatment, a 1.5-day increase in survival corresponds to survival of one-eighth as many cells, etc.

with theophylline (the validity of this interpolation is supported by the data in Table 4). A therapeutic index may be expressed as the ratio of the LD$_{50}$ to the 50% effective dose, which for BCNU alone is 44/33 = 1.3 and for BCNU given with theophylline is 27/8 = 3.4.

Mechanism of Synergistic Effect. As a first step toward elucidating the mechanism of interaction of BCNU and theophylline, we studied the uptake of $[^{14}C]$BCNU by L1210 cells treated with theophylline compared with the uptake of cells not receiving theophylline (Chart 4). Uptake was not significantly affected by theophylline pretreatment.

The timing of elevation of cAMP levels and the optimal timing of BCNU and theophylline treatment were studied. As shown in Chart 5, cellular cAMP reached a peak 1 hr after theophylline, was still modestly elevated at 2 hr, and returned to control levels at 4 hr. Similar relationships were observed when expressed on a cell number or protein content basis. The effect of timing of BCNU and theophylline on survival of mice bearing leukemia L1210 is shown in Table 5. If theophylline was given 1 or 2 hr before BCNU or concurrently, maximum antileukemic effects were seen. However, if theophylline was given at any time after BCNU, the effects were comparable to those for
DISCUSSION

Our studies support the conclusion that the cytotoxic effect of BCNU for both normal and leukemic cells is enhanced by treatment with theophylline, either preceding or concurrent with BCNU. However, the sensitizing effect for normal cells is less than that for leukemic cells, so that the therapeutic index is improved for the combined treatment versus BCNU alone. The sensitizing effect for normal cells is of the order of 2-fold, based on the ratio of blood counts (Table 3) and on the ratio of BCNU doses giving lethal toxicity (Chart 3). The LD$_{50}$ for BCNU alone (read from the curve in Chart 3) was 44 mg/kg and the LD$_{50}$ for BCNU in the theophylline-treated mice was 27 mg/kg for leukemic mice, which gives a LD$_{50}$ ratio of 44/27 = 1.6.

Estimating the sensitizing effect on leukemic cells is more complex because theophylline per se reduces the leukemic cell count. Comparing assays based on cell counts, we have a surviving fraction of 0.52 (Table 2) for theophylline alone. Pooling the data for theophylline plus BCNU in Tables 1, 2, and 4, we have 5 of 40 mice surviving after BCNU and 34 of 38 mice surviving after BCNU plus theophylline. Using Poisson statistics, this translates into an estimate of 2.0 surviving leukemia cells for BCNU alone and 0.12 surviving leukemia cells for BCNU plus theophylline (Table 6). The surviving fraction attributable to theophylline then becomes 0.12/2.0 = 0.06. The sensitizing ratio then becomes the ratio of the effect of theophylline alone versus theophylline in the combination, or 0.52/0.06 = 8.7.

The sensitizing effect may also be estimated from survival time data as follows. The 1-day increase in median survival for theophylline alone (data in Table 1 and confirmed in replicated experiments) leads to an estimated 0.25 surviving fraction (doubling time, 12 hr). The effect of theophylline when combined with BCNU may be estimated from a comparison of the median survivals for the combination versus BCNU alone. From the data in Tables 1 and 4, a conservative estimate is 3 days, which translates into a surviving fraction of 0.015. The sensitizing ratio is then 0.25/0.015 = 16.6 (Table 6). The reason this estimate is larger than the estimate based on cell numbers may be related to cell growth rate, as discussed below.

The magnitude of this sensitizing effect seems to be related
to the dose of BCNU (Table 4, Line 1 versus Line 2), and therefore the comparison of the equieffective doses (Chart 3) will be an underestimate of the sensitizing effects of the optimum dose of BCNU. From Chart 3, the dose necessary for 50% 30-day survivors was 33 mg/kg for BCNU alone and only 8 mg/kg for BCNU with theophylline, for a ratio of 33/8 = 4.1. This ratio obtained with a BCNU dose of 8 mg/kg is lower than the ratios of 8.7 and 16.6 noted above, which were determined at a BCNU dose of 20 mg/kg. Overall, the sensitizing effect for normal tissues is approximately 2-fold, whereas for leukemic cells the sensitizing effect is approximately 8-fold.

The observation noted above that the antileukemic effect estimated by comparison of survival is greater than the estimate based on the Poisson statistic suggests that cells which survived this combined treatment may have a slower growth rate than untreated cells. A slower growth rate after theophylline alone is also seen in the data in Chart 1. Such a reduction in cell growth rate suggests a change in one of the biological characteristics of theophylline-treated cells. Alterations of other biological characteristics of cells with cAMP stimulation have been reported by others (2–4, 8, 9, 13–16). Thus, the combination may have synergistic effects because of interaction of the combination at the time of treatment plus continued slowing of cell growth rate after treatment.

An interesting observation was the regrowth of leukemia cells in spite of continued theophylline treatment (Chart 1). This is supported by other experiments in our laboratory in which a single treatment with theophylline is nearly as effective as multiple treatments both in terms of cell kill and median survival. One explanation is that the leukemia cell population may be heterogeneous with respect to response to elevation of cAMP levels. A sensitive subpopulation may be killed while a resistant population may survive. Resistant subpopulations have been observed by others but required several transplant generations for separation of sensitive and resistant cell lines (3). An alternative explanation is that the initial rise in the cAMP level (Chart 5) may induce increased cellular levels of phosphodiesterase activity, which would then render cells less sensitive to phosphodiesterase inhibition by theophylline. Phosphodiesterase activity rises after cells are exposed to treatments that raise cAMP levels (6, 11). This change represents new enzyme synthesis caused by action of cAMP at the transcriptional level (12). This hypothesis of increased cellular phosphodiesterase activity after an initial rise in the level of cAMP would explain the results in Chart 1 as follows. In some cells (about 80%), theophylline treatment increases cAMP to a lethal level, and the cell dies. In other cells, an initial increase in cAMP stimulates synthesis of phosphodiesterase, which rapidly lowers the level of cAMP, and the cell survives. The refractoriness to subsequent theophylline treatment may then be explained on the basis of the increased phosphodiesterase activity. These 2 possible explanations are currently under further study.

We postulate that the mechanism for the sensitizing of cells to BCNU is mediated through elevation of cAMP levels. This is supported by the parallel dose response of cellular cAMP and the antileukemic effect in the study with different theophylline doses (Table 2). The study of timing of theophylline and BCNU also supports this mechanism. If BCNU is given 0 to 2 hr after theophylline, maximum cellular levels of BCNU will coincide with elevated cellular cAMP levels. Additional evidence is a parallel between the results of the time-sequence experiment reported herein and an experiment of similar design using dbcAMP. Thus, 2 of the 3 criteria for establishing the role of cAMP in a biological process proposed by Sutherland have been met (19). We appreciate that these correlations do not fully establish a cause-and-effect relationship and that other studies of mechanism are needed.

Alternate explanations for the effect of theophylline on the cell sensitivity to BCNU merit discussion. One possible mechanism would be based on methylxanthines interfering with mechanisms for repair of DNA. However, this does not seem to fit with the current results as follows. DNA repair occurs for several hr after chemotherapy damage (7); hence, one would have expected theophylline to have had an effect when given concurrently with BCNU or for several hr afterwards rather than the time course observed. Another possible mechanism would be that of theophylline being incorporated into the DNA and RNA of tumor cells. This has been reported for hamster melanoma cells (18). These authors report that part of the incorporated precursor may be in the form of novel nucleosides. Our results for time relationships would be consistent with this mechanism, but this would not explain the results for dbcAMP. We also considered that theophylline could alter cellular uptake of BCNU, but this seems to be excluded by the results in Chart 4. Thus, we believe our results are best explained via a modulation of cellular cAMP levels.

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Table 6

<table>
<thead>
<tr>
<th>Type of assay</th>
<th>Surviving fraction for theophylline alone</th>
<th>Surviving cells</th>
<th>BCNU alone</th>
<th>BCNU + theophylline</th>
<th>Theophylline effect</th>
<th>Estimate of sensitizing ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cells</td>
<td>0.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BCNU alone</td>
<td>0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.12/2.0 = 0.06</td>
<td>0.52/0.06 = 8.7</td>
<td></td>
</tr>
<tr>
<td>Survival time</td>
<td>0.25&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>0.015&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.25/0.015 = 16.6</td>
<td></td>
</tr>
</tbody>
</table>

*<sup>a</sup> Ratio of effect of theophylline alone (Column 2) to that of theophylline in combination with BCNU (Column 3).
*<sup>b</sup> From Table 2, Column 3, Line 4.
*<sup>c</sup> Based on pooling data in Tables 1, 2, and 4 and applying Poisson statistics.
*<sup>d</sup> Based on Table 1, Column 3, Lines 1 and 2.
*<sup>e</sup> Based on a 3-day increase in median survival (Tables 1 and 4).

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What are the clinical implications of these results? Cancer patients may be taking other medications (which may elevate cAMP levels) concomitantly with anticancer drugs and thus may inadvertently have an increased cytotoxic effect from the administered chemotherapy. It is noteworthy that the cytotoxic effect is proportionate to the dose of the drug that may elevate cAMP levels and is seen only when that drug is given prior to the cytotoxic drug. Thus, if one wishes to avoid unpredictably increased toxicity to normal cells, one could proscribe, prior to administration of chemotherapy, medications that elevate cAMP levels. Exploitation of the synergistic killing of tumor cells will require further study before clinical application. For example, it will be necessary to define the range of chemotherapy drugs for which this phenomenon applies, since anticancer drugs are often used in combinations in treating patients. Also, it would be of interest to define the cell types for which this phenomenon applies and to identify the drug(s) giving maximum elevation of cAMP levels in tumor cells with the least effect against normal cells. These studies are currently in progress.

ACKNOWLEDGMENTS

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REFERENCES

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