Antiangiogenic Chemotherapeutic Agents: Characterization in Comparison to Their Tumor Growth Inhibition in Human Renal Cell Carcinoma Models

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ABSTRACT

The mechanism of action of anticancer chemotherapeutic agents is mainly thought to be due to a direct inhibition of tumor cell proliferation. The enhanced endothelial cell proliferation rate in tumor specimens raised the question of whether therapeutic effects of chemotherapeutic agents might be at least partially attributed to inhibition of tumor angiogenesis. In the present study, we investigated the potential effects of chemotherapeutic agents on human renal carcinoma angiogenesis with the alginate implantation model in mice. For the first time, we also compared results from the angiogenesis model with the inhibitory effects on growth of s.c. xenografts in nude mice. Vincristine and bleomycin exerted strong inhibition of tumor angiogenesis in both carcinoma lines close to the level of the standard antiangiogenic agent O-chloroacetyl-carbamyl-fumagillol (AGM-1470; T/C 22%) but had no effect on Caki-1 angiogenesis (T/C 137%). Etoposide and 5-fluorouracil reduced Caki-1 tumor angiogenesis but had no effect on Caki-2. Despite antiangiogenic effects in both carcinoma lines, vincristine, bleomycin, and AGM-1470 significantly reduced only the growth of fast-growing Caki-1 s.c. xenografts but not the slow-growing Caki-2. Antivascular effects by bleomycin and AGM-1470 were also shown by a decrease of microvessel density in nude mouse xenografts. Our findings suggest that chemotherapeutic agents may exert inhibition of tumor angiogenesis, which could be exploitable by combination therapy of fast-growing tumors. The resistance of the slow-growing Caki-2 carcinoma against acute angiogenesis inhibition indicates a need for well-tolerated angiogenesis inhibitors. Our results also suggest the use of fast-growing s.c. xenografts for demonstrating growth inhibition by antiangiogenic compounds. Further characterization of antiangiogenic compounds considered for clinical application should, however, have its focus on slow-growing tumors, which are not accessible for most therapeutic strategies.

INTRODUCTION

Present antitumor therapies, i.e., the use of chemotherapeutic agents or radiation, have been optimized toward inhibition of tumor cell proliferation. Ongoing search for new chemotherapeutic agents specifically considers compounds with selectivity to a certain tumor entity and less inhibitory effects at proliferation of host cells. Besides new, promising chemotherapeutic agents directed on autonomous proliferation of tumor cells, inhibition of tumor blood supply has been realized as a unique approach to stop tumor growth (1, 2). Results from animal experiments indicated strong suppression of tumor growth and metastasis by specific inhibition of angiogenic growth factor action (3). Moreover, the proliferation rate of endothelial cells within tumor tissue gave rise to the assumption that antiangiogenic treatments might permit improved selectivity to the tumor (4, 5).

The conceptional basis of tumor angiogenesis has initiated a search for both antagonists of tumor angiogenesis induction (3, 6) and cytotoxic agents with high specificity to the endothelial cell (7, 8). The latter therapeutic opportunity suggests, however, that a hidden “antivascular” effect may be operating in a number of conventional anticancer therapies (5, 9, 10). This presumption has been implied by three observations: (a) an increased labeling index of tumor endothelial cells close to the level of tumor cells (11, 12); (b) direct damage of endothelial cells or the tumor endothelium (4, 13); and (c) selective inhibition of vascularization in the chorioallantoic membrane assay or cellular assays of angiogenesis by certain chemotherapeutic agents (14, 15).

More recent data on the proliferation activity of tumor endothelial cell in human tumors revealed evidence that endothelial cell proliferation was overstated in previous studies (16). By direct immunohistochemistry of tumor tissue, it was demonstrated that the labeling index of endothelial cells exerts only a 2- or 3-fold proliferation difference compared with normal tissue. However, significant endothelial cell labeling was seen at the periphery of the tumors, where tumor angiogenesis is most active (16).

In animals, direct morphological and ultrastructural damage to the endothelium has been seen in the tumor vasculature after intravascular administration of chemotherapeutic drugs. These studies showed capillary collapse and hemorrhagic necrosis due to swelling and disruption of the endothelium (13). Among a variety of chemotherapeutic agents frequently used in the clinic, bleomycin and Vinca alkaloids exerted the most toxic...
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Measurement of FITC-dextran accumulation [µg/alginate implant] by encapsulated tumor cells (17-19). Here we demonstrate that effects on the endothelium were predominantly present in the tumor vasculature. Investigation with the chorioallantoic membrane assay also demonstrated inhibitory effects on the developing vasculature. However, the reported experimental results do not allow conclusions to be drawn concerning the extent to which potential antivascular effects contribute to tumor growth inhibition.

In this report, we demonstrate potential antiangiogenic effects of chemotherapeutic agents in comparison to their effects on the growth of s.c. xenotransplants on nude mice. For determination of tumor angiogenesis, we used the murine alginate implantation model in which angiogenesis is selectively driven by encapsulated tumor cells (17-19). Here we demonstrate that anticancer chemotherapeutic agents, specifically, vincristine and bleomycin, can potently inhibit angiogenesis induction in two renal carcinoma lines, Caki-1 and Caki-2. We also found that tumor growth inhibition, potentially resulting from antiangiogenic effects by chemotherapeutic agents, depends on the growth kinetic of s.c. xenografts.

MATERIALS AND METHODS

Animals. Female inbred Swiss nu/nu mice weighing about 25 g were obtained from Charles River (Sulzfeld, Germany). Animals were purchased as specific pathogen free and received water and food ad libitum.

Materials. Sodium alginate of low viscosity and FITC-dextran, with an average molecular weight of 150,000, were purchased from Sigma Chemical Co. (Deisenhofen, Germany). FITC-dextran was dissolved in saline to a final concentration of 1%. AGM-1470 was kindly provided by Berlex Biosciences (San Francisco, CA). Cyclophosphamide, vincristine, bleomycin, Adriamycin, 5-fluorouracil, and etoposide were obtained from Sigma.

Tumor Cells. The human renal cell carcinoma lines Caki-1 and Caki-2 were obtained from American Type Culture Collection (Rockville, MD). Tumor cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1 mM sodium pyruvate.

Preparation of Alginite Beads Containing Tumor Cells. The method used for cell encapsulation was described in detail previously (17, 20). Briefly, sodium alginate of low viscosity was dissolved in sterile saline to a final concentration of 1.5%. Tumor cells were harvested from cell culture at 60–80% confluence. After centrifugation, the tumor cell pellet was directly resuspended with the alginate solution to the desired cell number and thereafter filled into a reservoir. Droplets containing tumor cells were produced by extrusion of the alginate solution through a 12-gauge cannula. The tumor cell alginate solution was dropped into a swirling bath of 80 mM CaCl\textsubscript{2}. The calcium ions caused immediate gelling of each droplet by an exchange of sodium from the alginate. The size of the beads was minimized by a laminar air flow along the cannula. After incubation in the CaCl\textsubscript{2} bath for an additional 30 min, the beads were washed twice with buffer, centrifuged, and prepared for injection. All procedures were carried out under sterile conditions at 37°C.

Angiogenesis Model. Female Swiss nu/nu mice were s.c. injected with 0.1 ml of alginate beads containing 3 × 10\textsuperscript{6} tumor cells into the upper third of the back. Cyclophosphamide (100 mg/kg), Adriamycin (7.5 mg/kg), vincristine (1.2 mg/kg), bleomycin (50 mg/kg), 5-fluorouracil (50 mg/kg), etoposide (20 mg/kg), and AGM-1470 (60 mg/kg) were injected i.p. on days 4 and 5 after alginate implantation. At the end of the experiment (day 14), 0.2 ml of 1% FITC-dextran solution (100 mg/kg) was injected i.v. into the lateral tail vein of mice. Alginate implants were rapidly removed 20 min after FITC-dextran injection, weighed, and transferred to tubes containing 2 ml of saline. The tubes were mixed by a vortex for 20 s and centrifuged (3 min; 1000 g). After dilution (1:1), the fluorescence of the supernatant was measured with a fluorescence spectrophotometer (Spectrofluor JY3 D; Jobin Yvon S.A. GmbH, Unterhaching, Germany) with excitation at 492 nm and emission at 515 nm. All experiments were performed in duplicate.

Nude Mouse Xenografts. For determination of the effects on xenotransplant growth, Swiss nu/nu mice were s.c. injected with 1 × 10\textsuperscript{6} tumor cells. Tumor growth was determined by caliper measurement of the largest diameter and its perpendicular. Tumor size was calculated as:

\[ \text{Tumor volume (mm}^3\text{)} = 0.5 \times a \times b^2 \]

where \( a \) is the largest diameter and \( b \) is its perpendicular.

Cyclophosphamide (100 mg/kg), Adriamycin (7.5 mg/kg),

\[ \text{AGM-1470}^3 \text{, O-chloroacetyl-carbamyl-fumagillol; MVD, microvessel density.} \]
Table 1  Effect of standard chemotherapeutic agents on angiogenesis of Caki-1 or Caki-2 angiogenesis in the alginate model
Female nude mice were implanted with 0.1 ml alginate beads containing 3 × 10³ Caki-1 human renal carcinoma cells. At least eight mice were allocated to each group. Chemotherapeutic drugs were applied at indicated doses on days 4 and 5 after alginate implantation. Quantification of tumor angiogenesis was performed on day 14 after alginate implantation. Effects on tumor angiogenesis are represented by FITC-dextran accumulation at the alginate implant (mean ± SE).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Caki-1</th>
<th>Caki-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1.9 ± 0.4</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>CTX</td>
<td>100 mg/kg</td>
<td>2.0 ± 0.5</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>ADR</td>
<td>7.5 mg/kg</td>
<td>2.6 ± 0.4</td>
<td>0.5 ± 0.1b</td>
</tr>
<tr>
<td>VCR</td>
<td>1.2 mg/kg</td>
<td>1.0 ± 0.2a</td>
<td>0.4 ± 0.1b</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>3.1 ± 0.3</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>BLEO</td>
<td>50 mg/kg</td>
<td>0.8 ± 0.2ab</td>
<td>0.8 ± 0.1b</td>
</tr>
<tr>
<td>5-FU</td>
<td>50 mg/kg</td>
<td>1.0 ± 0.2ab</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>ETP</td>
<td>20 mg/kg</td>
<td>1.0 ± 0.2ab</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>AGM</td>
<td>60 mg/kg</td>
<td>0.7 ± 0.1b</td>
<td>0.2 ± 0.02ab</td>
</tr>
</tbody>
</table>

*CTX, cyclophosphamide; ADR, Adriamycin; VCR, vincristine; BLEO, bleomycin; 5-FU, 5-fluorouracil; ETP, etoposide; AGM, AGM-1470.

RESULTS

Angiogenesis Induction.  s.c. implantation of alginate-encapsulated tumor cells and quantification of functional blood flow by the blood pool agent FITC-dextran provides a highly sensitive measurement of tumor angiogenesis (17). Implantation of alginate beads containing 3 × 10³ Caki-1 or Caki-2 human renal carcinoma cells induced an approximate 6-fold and 10-fold increase of angiogenesis, respectively, within 14 days (Fig. 1). We found no statistically significant difference in angiogenesis induction with either Caki-1 or Caki-2 cells.

Antiangiogenic Effects of Chemotherapeutic Agents.  Potential antiangiogenic effects of chemotherapeutic agents were tested by treatment of mice bearing alginate implants. Chemotherapeutic agents were applied on days 4 and 5 at doses known for potential tumor growth inhibition. All dosages were close to the maximum tolerated dose, as indicated by an impairment of body weight gain (data not shown). Angiogenesis induction of the Caki-1 renal carcinoma was inhibited by vincristine (TIC 52%), 5-fluorouracil (TIC 32%), etoposide (TIC 32%), and bleomycin (TIC 26%; Table 1). Cyclophosphamide and Adriamycin remained without effects on Caki-1 tumor angiogenesis. Tumor angiogenesis of the Caki-2 renal carcinoma line was reduced by Adriamycin (TIC 33%), bleomycin (TIC 50%), and vincristine (TIC 26%; Table 1). Cyclophosphamide, etoposide, and 5-fluorouracil did not reduce Caki-2 tumor angiogenesis (Table 1). Among the chemotherapeutic agents tested, vincristine and bleomycin led to a consistent reduction of tumor angiogenesis in both carcinoma lines. Tumor angiogenesis inhibition with 5-fluorouracil, etoposide, and Adriamycin was dependent on the tumor line. In addition, the standard antiangiogenic agent AGM-1470 led to a significant inhibition in both renal carcinoma lines (Table 1).

Tumor Growth Inhibition of Chemotherapeutic Agents.  Growth kinetics of both Caki-1 and Caki-2 human renal carcinomas after s.c. implantation into nude mice are shown by Fig. 2. The average tumor volume doubling time of Caki-1 and Caki-2 s.c. xenografts was 5.6 and 12.7 days, respectively. The growth-inhibitory action on s.c. xenografts was tested with identical dosages at the start of treatment, when primary tumors reached their exponential growth phase at a tumor volume of 50–100 mm³ (Fig. 2). Established Caki-1 human renal carcinomas were growth inhibited by all chemotherapeutic agents tested and the standard antiangiogenic com-
Tumor angiogenesis and Chemotherapy

Female nude mice were implanted s.c. with 10^6 Caki-1 or Caki-2 human renal carcinoma cells. Chemotherapeutic drugs were applied at the indicated doses for 2 days, starting when primary tumors had a mean tumor volume of 50–100 mm^3. At least eight mice were allocated to each group. Tumor volume (mean ± SE) at the start of treatment (day 0) and on day 12 after the start of treatment is represented. Treatment efficacy was calculated by determination of \( \Delta T \) (change of tumor volume between day 0 and day 12 in the treatment group) and \( \Delta C \) (change of tumor volume between day 0 and day 12 in the control). The degree of tumor growth inhibition can be obtained from \( \Delta T/\Delta C \).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Caki-1</th>
<th>Caki-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Size (mm^3)</td>
<td>Size (mm^3)</td>
</tr>
<tr>
<td>Control</td>
<td>86 ± 20</td>
<td>304 ± 61</td>
</tr>
<tr>
<td>CTX 100 mg/kg</td>
<td>86 ± 25</td>
<td>193 ± 41</td>
</tr>
<tr>
<td>ADR 7.5 mg/kg</td>
<td>90 ± 21</td>
<td>167 ± 28</td>
</tr>
<tr>
<td>VCR 1.2 mg/kg</td>
<td>89 ± 30</td>
<td>162 ± 36</td>
</tr>
<tr>
<td>Control</td>
<td>54 ± 12</td>
<td>197 ± 37</td>
</tr>
<tr>
<td>BLEO 50 mg/kg</td>
<td>55 ± 11</td>
<td>122 ± 28</td>
</tr>
<tr>
<td>5-FU 50 mg/kg</td>
<td>51 ± 9</td>
<td>98 ± 12</td>
</tr>
<tr>
<td>ETP 20 mg/kg</td>
<td>52 ± 8</td>
<td>139 ± 24</td>
</tr>
<tr>
<td>AGM 60 mg/kg</td>
<td>56 ± 11</td>
<td>128 ± 20</td>
</tr>
</tbody>
</table>

\(^a\) CTX, cyclophosphamide; ADR, Adriamycin; VCR, vincristine; BLEO, bleomycin; 5-FU, 5-fluorouracil; ETP, etoposide; AGM, AGM-1470.

\(^b\) \( p < 0.05 \), versus control.

DISCUSSION

As an important finding during the last years, it has become clear that tumor angiogenesis plays a pivotal role in progression of malignant tumors. The possible impact of standard chemotherapeutic agents on this process, however, remains unclear. Using the alginate angiogenesis model in mice, we: (a) determined angiogenesis inhibition by standard chemotherapeutic agents; and (b) correlated antiangiogenic effects and tumor growth inhibition of s.c. xenografts by these agents. We have carefully chosen two renal carcinoma lines, Caki-1 and Caki-2, for our investigation because these cell lines differ in growth as well as in morphology after xenografting onto nude mice (22–24). Caki-1 gives rise to poorly differentiated clear cell G3 tumors with a low tumor volume doubling time, whereas Caki-2 leads to well-differentiated clear cell G1 tumors with a high tumor volume doubling time (24). More importantly, however, both tumors demonstrated comparable angiogenic potencies, irrespective of their histological phenotype and growth rates.

The results of the present study demonstrate, for the first time, inhibitory effects of antineoplastic chemotherapeutic agents in an in vivo model of tumor angiogenesis. Moreover, we could group antiangiogenic effects according to two patterns of action, depending on the consistency of angiogenesis inhibition in both lines. The first “type” (i.e., bleomycin and vincristine) is defined by a potent angiogenesis inhibition, regardless of the cell line used. It seems conceivable to assume that “type 1” chemotherapeutic agents interfere with the mitogen-activated host vasculature. Our conclusions on the type 1 agents bleomycin and vincristine are in accord with previous studies, which revealed evidence for direct damage of the host vasculature. Bleomycin is known to induce dose-dependent pulmonary fibrosis in humans as well as in animals (4, 25). Morphologically, endothelial retraction, swelling, and disruption of the endothelium can be observed (4, 26). Identi cal toxic effects on tumor vasculature have been described for Vinca alkaloids. Up to 80% decrease of tumor blood flow was obtained with Vinca alkaloids in different mouse tumors (13, 27). This effect seems to be responsible for hemorrhagic necrosis, which was first described in the Colon 38 mouse tumor (28).

The second “type” of reaction patterns (i.e., Adriamycin, 5-fluorouracil, and etoposide) seems to inhibit angiogenesis via a direct inhibitory action on the tumor cell; angiogenesis inhibition by chemotherapeutic agents was only evident in one of the two renal carcinoma lines, and none was active in both lines. We assume that an inhibitory action on the tumor cell itself results into an inability to release angiogenic growth factors or proteases. However, cytotoxic effects on
tumor cells resulting in growth inhibition of xenografts can appear without demonstration of angiogenesis inhibition. For example, the alkylating agent cyclophosphamide, also known for toxic effects on the endothelium (29), failed to exert angiogenesis inhibition with both Caki-1 and Caki-2 renal carcinoma, whereas the growth of Caki-1 xenografts was suppressed.

In addition to the alginate model in mice, which provides information on antiangiogenic effects of investigational compounds (17), we also determined MVD, a well-established parameter of tumor vascularity. All chemotherapeutic agents tested in our panel decreased the MVD of Caki-1 xenografts, even cyclophosphamide and Adriamycin, which inhibited primary tumor growth but remained without effects in the alginate angiogenesis model. This result further suggests that antivasculare effects may arise from direct cytotoxic effects on tumor cells. In contrast, the MVD of Caki-2 xenografts was only reduced by bleomycin and AGM-1470, whereas vincristine and Adriamycin (both active in the alginate angiogenesis model) failed to reduce MVD. Histological analysis revealed, however, evidence for a significant hemorrhagic necrosis without affecting the MVD, based on determination of CD31-positive microvessels (data not shown). This finding proposes the use of the alginate angiogenesis model for testing antiangiogenic effects, because determination of functional blood volume is also sensitive to antivasculare effects based on hemorrhagic necrosis. Taken together, the determination of MVD after treatment of nude mouse xenografts further suggests two different "types" of antivasculare reaction patterns.

Besides different patterns of antiangiogenic action, both type 1 and type 2 anticancer chemotherapeutic agents led to a significant inhibition of the fast-growing Caki-1 xenografts. Slow-growing Caki-2 xenografts remained, however, resistant against antiangiogenic chemotherapeutic drugs. We interpret this finding as a pattern of differential dependence of tumor growth from blood supply. The breakdown of the supportive endothelium results in an immediate deprivation of nutritious factors necessary for survival of the tumor. This is reflected by an inhibition of fast-growing Caki-1 xenografts, whereas Caki-2 may have a competitive edge for survival because of their relatively slow growth kinetics. Our hypothesis is further supported by the type 1 compound AGM-1470, a prototype of a chemotherapeutic agent with high specificity for the growing endothelium (30–32). Similar to reported data on the growth-inhibitory potential (32), we observed tumor growth suppression with AGM-1470 in mice bearing Caki-1 s.c. xenografts. In contrast, slow-growing Caki-2 s.c. xenografts were not affected by AGM-1470, although angiogenesis induction in the alginate model was substantially suppressed.

In conclusion, the present results provide strong evidence for antiangiogenic effects of chemotherapeutic agents. We could group the pattern of action with respect to the consistency of angiogenesis inhibition in both lines: type 1, which is independent of the tumor line used; and type 2, which is dependent. Considering our results obtained from xenograft experiments, the antiangiogenic potential of type 1 chemotherapeutic agents could be exploitable in the clinic by combination therapy used for fast-growing tumors. The resistance of slow-growing tumors to acute antiangiogenic effects of the chemotherapeutic agents tested suggests the need for well-tolerated angiogenesis inhibitors with a large effective dose window. With respect to the identification and development of new antiangiogenic compounds, our results also imply the use of fast-growing s.c. xenografts for demonstrating growth inhibition by antiangiogenic compounds. Further characterization of antiangiogenic compounds considered for clinical application should, however, have its focus on slow-growing tumors, which have not been accessible for most therapeutic strategies.

**Table 3** Effect of standard chemotherapeutic agents on MVD in Caki-1 or Caki-2 nude mouse xenografts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>MVD/×10 field</th>
<th>MVD/×20 field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>39 ± 1.4</td>
<td>33 ± 1.6</td>
</tr>
<tr>
<td>CTX</td>
<td>100 mg/kg</td>
<td>26 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37 ± 2.1</td>
</tr>
<tr>
<td>ADR</td>
<td>7.5 mg/kg</td>
<td>25 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21 ± 4.1</td>
</tr>
<tr>
<td>VCR</td>
<td>1.2 mg/kg</td>
<td>23 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39 ± 9.1</td>
</tr>
<tr>
<td>BLEO</td>
<td>50 mg/kg</td>
<td>14 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14 ± 4.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5-FU</td>
<td>50 mg/kg</td>
<td>17 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34 ± 1.8</td>
</tr>
<tr>
<td>ETP</td>
<td>20 mg/kg</td>
<td>18 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24 ± 9.1</td>
</tr>
<tr>
<td>AGM</td>
<td>60 mg/kg</td>
<td>26 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14 ± 5.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> CTX, cyclophosphamide; ADR, Adriamycin; VCR, vincristine; BLEO, bleomycin; 5-FU, 5-fluorouracil; ETP, etoposide; AGM, AGM-1470.

<sup>b</sup> P < 0.05, versus control.

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Fig. 2 Growth kinetics of Caki-1 and Caki-2 after s.c. implantation of 10<sup>6</sup> cells into nude mice [means ± SE (bars); n = 8].
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REFERENCES

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