Enhancement of N-Nitrosodiethylamine-initiated Hepatocarcinogenesis Caused by a Colchicine-induced Cell Cycle Disturbance in Partially Hepatectomized Rats

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ABSTRACT

The effects of a colchicine-induced M-phase block of regeneration after partial hepatectomy on early-stage liver carcinogenesis were studied in rats. When administered 1 or 3 days after N-diethylaminoethylsarcosine initiation and partial hepatectomy, colchicine increased the mitotic index of regenerating hepatocytes at days 4–6 without evidence of liver cell necrosis. When the protocol was combined with a selection procedure (E. Cayama et al., Nature (Lond.), 275: 60–62, 1978), a significant increase in the size but not number of γ-glutamyltranspeptidase-positive foci at week 5 was observed in a colchicine dose-dependent manner. This was associated with an elevated incorporation of 5-bromo-2-deoxyuridine into the γ-glutamyltranspeptidase-positive cells. In a longer-term experiment, the numbers, sizes, and 5-bromo-2-deoxyuridine labeling index of persistent nodules were increased significantly in colchicine-treated rats at week 9. This was associated with significant increases in the incidences and numbers of hepatocellular carcinomas at week 42. The above results raise the interesting possibility that a cell cycle disturbance in the early stage of liver carcinogenesis provides a persisting growth advantage for initiated cells, resulting in enhanced growth of foci and persistent nodules that evolve into hepatocellular carcinomas.

INTRODUCTION

In recent years, the cell cycle and its control have become highlighted as research topics, and it has been established that key players in regulation of passage from one phase to the next constitute a universally operating system in eukaryotes (1). With progress in research into gene abnormalities in various cancers and the mechanisms of cell proliferation in normal cells, it has been suggested that deregulation of the cell cycle may be a key event for acquisition of malignant potential during the development of cancer cells (2–5). Furthermore, accelerated cell proliferation has been recognized to lead to a general increase in the population of cells susceptible to malignant transformation (6). The relevance of a cell cycle block, occurring in the early stage of carcinogenesis, to the induction of preneoplastic lesions and their evolution to neoplasms has, however, hitherto attracted only limited attention.

In the present study, colchicine was selected for investigation, because it is well known as a chemical that can induce a block of the cell cycle at M phase by inhibiting polymerization of tubulins and depolymerization of microtubules (7, 8). In the first experiment documented here, the blocking effects of colchicine on regenerating hepatocytes were confirmed. In the second and third experiments, short- and long-term studies of liver carcinogenesis using an initiation-selection protocol were performed. The results suggest that colchicine disturbance of the cell cycle may provide a useful experimental model for studying the mechanisms of growth advantage in rat liver carcinogenesis.

MATERIALS AND METHODS

Animals

Male Fischer 344 rats (Shizuoka Laboratory Animal Center, Shizuoka Japan), 5 weeks old and weighing 100–110 g at commencement, were used in the experiments. The animals were housed in stainless-steel, wire-bottom cages in an air-conditioned room at 25°C with a 12-h light-dark cycle. Food and water were given ad libitum throughout the experiments. After a 1-week acclimation period on a basal diet in pellet form (Oriental MF Diet; Oriental Yeast Co., Ltd., Tokyo, Japan), the animals were allocated to experimental groups.

Chemicals and Diets

DEN was purchased from Wako Pure Chemical Co., Ltd. (Kyoto, Japan) and diluted with a 0.9% NaCl solution to a concentration of 0.1%. Colchicine was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in a 0.9% NaCl solution to a concentration of 0.05%. AAF and CCl4 were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and the latter was diluted 1:1 with corn oil. Diet containing 0.02% AAF was prepared by admixing the chemical with Oriental MF powdered diet.

Experimental Protocols

Experiment 1: M-Phase Block of Hepatocyte Regeneration by Colchicine

The protocol used in this experiment is shown in Fig. 1A. The animals were divided into three experimental groups. All animals received a single i.p. injection of DEN at a dose of 10 mg/kg body weight and underwent PH (9) 4 h thereafter. Group 1 then received an i.p. injection of saline, and groups 2 and 3 received i.p. injections of colchicine at doses of 0.25 and 0.5 mg/kg body weight, respectively, 1 and 3 days after the beginning of the experiment. Rats were sacrificed under light ether anesthesia on days 4, 5, or 6. Blood was sampled from the aorta for the assessment of serum activity of ALT, and livers were immediately excised, weighed, and processed for histological examination.

Experiment 2: Effects of the M-Phase Block on Induction of Enzyme-altered Foci

The experimental protocol is shown in Fig. 1B. The animals were divided into nine groups. Groups 1–6 received an i.p. injection of DEN at a dose of 10 mg/kg body weight, and groups 7 to 9 were administered the saline vehicle. After 4 h, all rats underwent PH; at 1 and 3 days, rats from groups 1, 5, and 7 received i.p. injections of saline and rats from groups 2, 3, 4, 6, 8, and 9 received i.p. injections of colchicine at doses of 0.1, 0.25, 0.5, 0.5, 0.25, and 0.5 mg/kg body weight, respectively. After an 11-day recovery period, all rats except those of groups 5 and 6 were placed on the selection regimen, comprising feeding of 0.02% AAF diet for 2 weeks and a single i.g. administration of CCl4 at 1 ml/kg body weight midway, following the procedure described by Cayama et al. (10). All animals were killed under ether anesthesia and the livers excised 5 weeks after the beginning of the experiment. Rats from groups 1 and 4 received an i.p. injection of BrdUrd (Sigma Chemical

3 The abbreviations used are: DEN, N-nitrosodiethylamine; AAF, 2-acetylaminofluorene; ALT, alanine aminotransferase; BI, N-benzyl imidazole; BrdUrd, 5-bromo-2-deoxyuridine; GGT, γ-glutamyltranspeptidase; HCC, hepatocellular carcinoma; LI, labeling index; MI, mitotic index; PB, phenobarbital; PH, two-thirds partial hepatectomy; γT, intragastric.
Co.) at a dose of 200 mg/kg body weight 2 h before sacrifice. Numbers and sizes of foci were examined using GGT staining as a marker.

Experiment 3: Evolution of Enzyme-altered Foci through Persistent Nodules to HCCs. The experimental regimen is shown in Fig. 1C. Groups 1 and 2 received the same procedures, respectively, as groups 1 and 4 of experiment 2; after an additional 4 weeks on basal diet, they were sacrificed. The animals received an i.p. injection of BrdUrd at 200 mg/kg body weight 2 h before sacrifice, and the numbers and sizes of persistent and remodeling nodules were examined using GGT staining as a marker.

Groups 3, 4, 5, and 6 received the same procedures, respectively, as groups 1, 4, 7 and 9 of experiment 2 and were then maintained on basal diet until sacrifice at week 42. Numbers and histological grades of neoplastic lesions were examined using H&E staining.

Histological Examination. For experiments 1–3, one slice per excised liver lobe was fixed in 95% ethanol containing 1% acetic acid at 4°C for 2 h followed by 99.5% ethanol at 4°C overnight, and processed routinely for paraffin embedding, sectioning at a thickness of 5 µm, and staining with H&E. In experiment 1, hepatocytes in M phase were counted and the MI expressed as the number of cells in M phase per 1000 hepatocytes counted. In experiment 2, HCCs were diagnosed histologically according to the criteria of Squire and Levitt (11).

Histochimical and Immunohistochemical Examinations. Section of livers of all rats from experiment 2 and groups 1 and 2 from experiment 3 were stained histochemically for GGT by the method of Rutenberg et al. (12). In experiment 2, GGT-positive foci were defined as rounded areas homogenously stained with GGT. In groups 1 and 2 from experiment 3, nodules stained homogenously with GGT were designated as persistent and those only stained patchily with GGT as remodeling (representative features are shown in Fig. 3), as described earlier by Tatematsu et al. (13). For quantitative assessment of lesion development, the numbers and areas of GGT-positive lesions were analyzed with the aid of an image analyzer, model HYB-C995 (Hamamatsu Television Co., Ltd, Shizuoka, Japan) connected to a Hewlett-Packard Desktop Computer System 45. The numbers of GGT-positive lesions per cm² were calculated using the formula of Campbell et al. (14). For the assessment of hepatocyte proliferation in GGT-positive lesions, serial liver sections were incubated with mouse anti-BrdUrd monoclonal antibody (Becton Dickinson Immunocytometry Systems, Mountain View, CA) at a dilution of 1:100 by the avidin-biotin-peroxidase complex method (15). Cells were considered positive for BrdUrd when clear reddish-brown staining of the nucleus could be identified. The BrdUrd LI of GGT-positive foci was assessed by counting BrdUrd-positive nuclei of hepatocytes stained for GGT with comparative observation of the GGT-stained serial sections for orientation. In each rat, the BrdUrd LI was determined by counting a total of 5000 hepatocytes in 10 randomly selected GGT-positive areas and expressed as the percentage of positive nuclei.

Mutation Assay. BI was obtained from Aldrich Chemical Co. (Milwaukee, WI) and PB was obtained from Wako Pure Chemical Co., Ltd., (Kyoto, Japan).

### Table 1 Mls and serum ALT activity after PH in experiment 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Colchicine (mg/kg)</th>
<th>Mls</th>
<th>Days after the beginning of the experiment</th>
<th>Serum ALT value</th>
<th>Liver weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>2.1±1.2</td>
<td>0.3±0.5</td>
<td>0.2±0.4</td>
<td>71±22</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>5.5±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5±1.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.6±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74±22</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>18.0±14.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.2±2.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.8±2.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>164±95</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means ± SDs.

<sup>b</sup> Each group consisted of five animals.

<sup>c</sup> Liver weights obtained at day 6 of the experiment.

<sup>d</sup> Significantly different from group 1.

### Table 2 Details of experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>DEN</th>
<th>Colchicine&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AAF+CCl&lt;sub&gt;4&lt;/sub&gt;</th>
<th>Effective no. of rats</th>
<th>Body weight (g)</th>
<th>Liver weight to body weight ratio (×10&lt;sup&gt;3&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>Saline</td>
<td>+</td>
<td>18</td>
<td>128.7±4.7</td>
<td>195.6±11.4</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>+</td>
<td>+</td>
<td>10</td>
<td>129.0±3.9</td>
<td>182.8±7.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>0.25</td>
<td>+</td>
<td>11</td>
<td>127.5±6.0</td>
<td>176.2±14.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>0.5</td>
<td>+</td>
<td>11</td>
<td>131.1±6.0</td>
<td>177.5±14.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>Saline</td>
<td>-</td>
<td>10</td>
<td>126.9±5.0</td>
<td>221.0±26.9</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>0.25</td>
<td>-</td>
<td>10</td>
<td>128.2±6.6</td>
<td>215.0±19.9</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>Saline</td>
<td>+</td>
<td>8</td>
<td>129.2±5.2</td>
<td>201.3±14.3</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>0.25</td>
<td>+</td>
<td>11</td>
<td>125.2±5.2</td>
<td>175.9±15.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>0.5</td>
<td>+</td>
<td>9</td>
<td>123.2±4.2</td>
<td>184.0±22.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means ± SDs.

<sup>b</sup> mg/kg body weight.

<sup>c</sup> Significantly different from group 1.

<sup>d</sup> Significantly different from group 7.
### Table 3 Numbers and areas of GGT-positive foci in rats in experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Effective no. of rats</th>
<th>No./cm³</th>
<th>Average size (mm²)</th>
<th>Percentage of liver area occupied by foci</th>
<th>BrdUrd LI in hepatocytes of foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>244 ± 107</td>
<td>0.3 ± 0.4</td>
<td>3.8 ± 2.0</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>289 ± 47</td>
<td>0.7 ± 0.5</td>
<td>18.6 ± 7.4ab</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>269 ± 56</td>
<td>1.1 ± 0.9a</td>
<td>31.2 ± 11.9b</td>
<td>n.d.</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>235 ± 90</td>
<td>1.5 ± 1.5b</td>
<td>44.9 ± 10.9b</td>
<td>4.2 ± 1.9b</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>17 ± 10</td>
<td>0.5 ± 0.4</td>
<td>0.4 ± 0.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>13 ± 10</td>
<td>0.4 ± 0.3</td>
<td>0.5 ± 0.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>19 ± 15</td>
<td>0.4 ± 0.4</td>
<td>0.3 ± 0.2</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* Values are means ± S.D.

* b Significantly different from group 1.

* c nd., not determined.

Effects of colchicine (0.01–50 mg) on the mutagenicity of 10 mg of DEN dissolved in water were also assessed in either the presence or absence of S9 mixture, using TA100 as the tester strain (16).

### Statistical Analysis

Quantitative differences between groups were statistically analyzed with the Student's t test. The $\chi^2$ test was applied to test the hypothesis that the frequency of HCC development was equal between groups in experiment 3. A probability value $P < 0.01$ was considered significant.

### RESULTS

#### MI Changes without Liver Cell Necrosis Caused by Colchicine in Experiment 1

MI and serum ALT values as a function of time and liver weight at day 6 after DEN and colchicine are shown in Table 1. MI values in group 1 decreased steadily from day 4 and reached the preoperative level at day 6. Significant increases in MI as compared with group 1 were detected in groups 2 and 3 at these time points. Histological examination revealed no marked liver cell damage or single-cell necrosis (apoptosis) in any of the three groups. Moreover, no significant differences in serum activities of ALT were observed from days 4—6. The remnant liver weights were significantly lighter in groups 2 and 3 than in group 1 on days 4, 5, and 6 (data for the last time point shown as representative figures).

#### Enhanced Growth Advantage of GGT-positive Foci Caused by Colchicine in Experiment 2

During the experimental period, average food intake was constant among groups (data not shown). Final body weights showed significant decreases in groups 2, 3, and 4, in which rats received DEN plus colchicine, as compared with group 1. In contrast,
the relative liver weights in group 4 were significantly heavier than those in group 1.

The numbers, areas, and BrdUrd LI for foci are summarized in Table 3. Groups 2, 3, and 4, which received 0.1, 0.25, and 0.5 mg/kg body weight of colchicine, respectively, exhibited significant increases in the size and/or the percentage of liver area occupied by foci in a dose-dependent manner. However, the numbers of GGT-positive foci per cm² were not significantly different among groups 1–4, which received DEN and the selection regimen. The BrdUrd LI in foci in group 4 was significantly higher as compared with group 1. Representative GGT-positive foci in the livers of rats from groups 1–4 are shown in Fig. 2. Very much larger foci developed in rats receiving DEN followed by colchicine.

Higher incidences of Persistent Nodules and HCCs in Experiment 3. The numbers, sizes of persistent and remodeling nodules, and BrdUrd LI of the former are shown in Table 4. The numbers of persistent nodules were significantly greater in group 2, whereas remodeling nodules increased in group 1, with totals being approximately equal in both groups. Persistent nodules of group 2 were significantly larger as compared with those of group 1. BrdUrd LIs in persistent nodules were also increased as compared with those in group 1. Representative persistent and remodeling nodules are shown in Fig. 3, clear differences being evident between the two groups. The incidences and numbers of HCCs at week 42 are shown in Table 5, with values significantly higher in group 4 than in group 3. Histologically, all HCCs showed were well or moderately differentiated carcinomas.

Lack of Mutagenicity of Colchicine. Fig. 4 shows the dose-response curves for toxicity and mutagenicity of colchicine in Salmonella typhimurium strain TA100 in the absence or presence of liver S9 from BI or PB-treated rats. In both cases, there was no increase in colonies on the plates with 50 mg of colchicine. Fig. 5 shows the colchicine dose-response curves obtained with strain TA100 with or without activation of 10 mg of DEN by liver S9 from BI-treated rats. DEN showed very weak mutagenicity in the liver S9, and no effect of additional colchicine was observed.

Tables

<table>
<thead>
<tr>
<th>Group</th>
<th>DEN</th>
<th>Colchicine (dose)</th>
<th>AAF + CCI₄</th>
<th>No. of rats</th>
<th>Persistent nodules</th>
<th>Remodeling nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No./cm² Size (mm²)</td>
<td>No./cm² Size (mm²)</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>11</td>
<td>11.1 ± 7.1 1.6 ± 1.3</td>
<td>1.4 ± 1.6 0.8 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>(0.5)</td>
<td>+</td>
<td>15</td>
<td>32.5 ± 6.7 4.6 ± 3.8</td>
<td>29.1 ± 14.5 3.5 ± 1.5</td>
</tr>
</tbody>
</table>

Values are means ± SDs.

*b mg/kg body weight.

Significantly different from group 1.

DISCUSSION

The first experiment showed clearly that induction of a cell cycle disturbance, the M-phase block, by colchicine after PH causes an...
HEPATOCELLULAR CARCINOGENESIS AND CELL CYCLE DISTURBANCES

A

Fig. 4. A. toxicity; B, mutagenicity of colchicine in S. typhimurium TA100 in the absence (△) or presence (C) of liver S9 from BI-treated Wistar rats. Survivors represent the percentage of 765–910 (TA100) bacteria that survived exposure to colchicine. Dotted line, number of spontaneous revertants.

B

Fig. 5. Effects of colchicine (0.01–50 mg) on the mutagenic activity of 10 mg of DEN in the absence (△) or presence (C) of liver S9 from BI-induced Wistar rats. Dotted line, number of spontaneous revertants.

carcinogenic process from the viewpoint of abnormalities of genes that could bring about loss of control (25–27). Such investigations have focused mainly on already developed preneoplastic lesions or neoplasms, and the emerging concept is that such cell cycle abnormalities are involved mainly in the late stages of neoplasia. To our knowledge, there has been no report of the effects of cell cycle abnormalities occurring in the initial stages of carcinogenesis. To shed light on this point, we therefore induced a disturbance of the cell cycle by colchicine in the present rat liver chemical carcinogenesis model. A proportion of the preneoplastic foci induced by the procedure of Cayama et al. (10) adopted in the present study are known to evolve finally into HCCs through a well-characterized sequence of lesions (28). Interestingly, drastically increased sizes of GGT-positive foci were observed in colchicine-treated groups as compared with the non-colchicine-treated group in experiment 2. It is thus conceivable that the cell cycle disturbance caused by colchicine differently affects hepatocytes initiated by DEN and their “normal” counterparts to give a growth advantage. That a persisting alteration is involved is indicated by the high BrdUrd LI in GGT-positive lesions in colchicine-treated groups in both experiments 2 and 3. Colchicine itself did not appear to initiate hepatocytes within the realms of the detectability in the present system, because the total numbers of foci remained constant, regardless of whether colchicine was injected or not in groups 1–4 in experiment 2. Furthermore, it is also supported by the mutation assay confirming no mutagenicity for colchicine or comutagenic effects with DEN. Groups 7–9 without DEN developed very small foci with no differences in size and number of lesions between groups. These foci may be recognized as an artifact of the selection regimen and support the possibility mentioned above.

Interestingly, the groups that received DEN with or without colchicine but not the selection regimen (groups 5 and 6) in experiment 2 lacked appreciable development of lesions in the liver. Therefore, it may be speculated that the proliferative advantage obtained because of the colchicine-induced cell cycle disturbance requires conditions of rapid growth to become evident.

At 9 weeks, considerable numbers of foci had evolved into persistent nodules in colchicine-treated groups, whereas the vast majority of lesions in the non-colchicine-treated group underwent remodeling. The latter process has been ascribed to a loss of the acquired altered phenotype accompanied by a gradual return to the normal-looking lobular architecture (13). It remains unclear what factors are involved in triggering and driving the remodeling process. However, the colchicine-induced cell cycle disturbance clearly exerted an influence,
contributing to persistence. This appeared to be linked to enhanced evolution of nodules into HCCs, as evidenced by the long-term findings.

The mechanism of action of colchicine in the present model is intriguing. Chemicals that inhibit mitosis such as colchicine or Colcemid can induce endoreduplication in regenerating cells (29, 30), possibly because of progression into a second S phase without undergoing cytokinesis, while cells are arrested in M phase (31). Endoreduplication can also be induced by other cell cycle-blocking agents (32). Moreover, cell cycle-blocking agents can also induce polyploidization in regenerating hepatocytes (23). It has been suggested that the existence of endoreduplicated or polyploid cells is well correlated with genomic instability, which might have been induced in initiated hepatocytes by the colchicine injections in the present study. Furthermore, gene amplification can be induced in regenerating cells by treatment with cell cycle blocking agents (33). It is thus conceivable that such cells are at high risk of developing genetic abnormalities. Colchicine is considered to have other kinds of action such as anti-inflammatory action, interfering with cell aggregation or antifibrotic action by the inhibition of microtubular functions (34, 35). Our colleagues recently reported (36) that anti-inflammatory agents prevent the preneoplastic lesion development as well as the appearance of liver cirrhosis using a rat hepatocarcinogenesis model. Thus, the contribution of the latter described actions of colchicine in the present study seems unlikely; however, the precise mechanism that led to the high proliferation of initiated cells documented clearly warrants additional study.

On the other hand, studies on epigenetic events related to the mechanisms of growth advantage per se (e.g., abnormal production of growth factors and their receptors or aberrant signaling pathways in the process of evolution from foci through persistent nodules into HCCs) are also required. Whatever the mechanism, the present results provide evidence for the importance of a cell cycle disturbance, an M-phase block, in the early stage of liver carcinogenesis, for eventual HCC development.

REFERENCES

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