Intracellular Hydroxyl Radical Production Induced by Recombinant Human Tumor Necrosis Factor and Its Implication in the Killing of Tumor Cells in Vitro

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ABSTRACT

This study investigated the effect of recombinant human tumor necrosis factor (rhTNF) on hydroxyl radical production by established cell lines in vitro, and its implication in the killing of tumor cells by rhTNF. During incubation of TNF sensitive mouse tumorigenic fibroblast L-M cells (2 × 10⁷ cells) in the presence of rhTNF (100 U), hydroxyl radical production as detected by the evolution of methane gas from dimethyl sulfoxide increased gradually, at 18 h reaching 1.8 times that in the absence of rhTNF. This increase was dependent on the concentration of rhTNF and was effectively prevented by the simultaneous addition of anti-rhTNF monoclonal antibody III 2F3, which inhibited both the binding of rhTNF to its receptor and the cytotoxic activity of rhTNF. The addition of iron chelator 2,2'-bipyridine, which inhibits iron-catalyzed Fenton reaction and so inhibits hydroxyl radical generation, suppressed both the increase of hydroxyl radical production and the cytotoxicity induced by rhTNF. A similar increase in hydroxyl radical production in the presence of rhTNF was also detected with TNF sensitive human myosarcoma-derived KYM cells, but no such increase was detected with TNF insensitive human embryonic lung fibroblast HEL cells. The results show that rhTNF induces increased hydroxyl radical production in TNF-sensitive cells, and suggest that this plays an important role in the mechanism of tumor cell killing by rhTNF.

INTRODUCTION

TNF is a macrophage/monocyte-derived anticancer cytokine (1-3) with strong cytotoxicity to tumor cells in vitro (4-10). The mechanism of its lethal effect on tumor cells, however, remains largely unexplained, although some studies have suggested the involvement of lysosomal enzyme (11, 12) and hydroxyl radical (12-14), based on the observation of suppressed TNF cytotoxicity in the presence of various inhibitors.

Various studies have shown that TNF cytotoxicity is suppressed in the presence of the hydroxyl radical scavengers dimethyl sulfoxide (DMSO) (12-14) and promethazine (14), and also in the presence of an iron chelator (desferal) (13, 14), which is known to block hydroxyl radical production. Such studies are methodologically limited, however, as cytotoxic suppression in itself does not provide a means of determining the kinetics of hydroxyl radical generation in TNF-sensitive cells, nor of detecting any changes in hydroxyl radical quantities in cells insensitive to TNF. It has thus not been possible to demonstrate a direct relationship between the cytotoxic effect of TNF and its stimulation of hydroxyl radical production.

We therefore attempted to measure the amount of hydroxyl radical produced in several cell lines under various conditions following their exposure to rhTNF and investigated the relation between changes in this production and the cytotoxic effects of rhTNF.

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The abbreviations used are: TNF, tumor necrosis factor; DMSO, dimethyl sulfoxide; rhTNF, recombinant human tumor necrosis factor; DETAPAC, diethylene triaminopentaaacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EMEM, Eagle's minimal essential medium; SOD, superoxide dismutase; FBS, fetal bovine serum.

MATERIALS AND METHODS

Materials. SOD, 2,2'-bipyridine, and DETAPAC were purchased from Sigma (St. Louis, MO); catalase from Boeringer Mannheim (W. Germany); DMSO and HEPES from Wako Chem. Co., Ltd. (Osaka). rhTNF and Monoclonal Antibody to rhTNF. rhTNF, produced in Escherichia coli and purified (99.9%) (15), and mouse anti-rhTNF monoclonal IgG antibody III 2F3 which neutralized the rhTNF activity (16) were generously provided by Asahi Chemical Ind., Co., Ltd. (Tokyo). The rhTNF had a specific activity of 2.3 × 10⁶ U/mg protein as determined by its cytotoxic activity against mouse L-M cells (16) and a molecular mass of 51,000 as trimer.

Radioiodination of rhTNF was performed by the method of Bolton and Hunter (17) using Bolton and Hunter reagent (4000 Ci/mmol, 148 TBq/mmol, NEN Research Products, Boston, MA) to obtain 125I-labeled rhTNF with specific activity of 250 cpm/fmol and no significant loss in bioactivity (18).

Cell Culture. L-M (mouse tumorigenic fibroblast) cells and HEL (human embryonic fibroblast) cells were obtained from the American Type Culture Collection, Rockville, MD. These cells were cultured in EMEM (Flow Lab., Australia) with 5% or 10% heat-inactivated FBS (Flow Lab.), 100 units/ml of penicillin, 100 µg/ml of streptomycin and 10 mm HEPES (pH 7.2) at 37°C in 5% CO₂ incubator. KYM cells derived from human myosarcoma were kindly provided by Dr. M. Sekiguchi (Cancer Research Institute, Tokyo University) and were cultured in DM-160 medium (Kyokuto Pharmaceutical Ind., Tokyo) with 10% FBS, antibiotics, HEPES and other conditions as above.

In Vitro Assay for Cytotoxic Action. L-M cells, (1 × 10⁷ cells/200 µl) were added to well of 96-well microculture plate (Sumitomo Bake-lite, Tokyo), and incubated at 37°C for 4 h in 5% CO₂. Medium was aspirated and both rhTNF (200 U/ml, 100 µl) and one of the agents indicated in Table 1 (100 µl) in EMEM were added, followed by incubation at 37°C for 24 h in 5% CO₂. The cytotoxic activity was then assessed by the dye uptake method using methylene blue (16).

Binding Assay. Binding assays were performed in triplicate on monolayers of L-M cells (5 × 10⁵ cells/well) in 12-well culture clusters (Costar, Cambridge, MA). The cells were incubated with 5 nM (586 U/ml) (125)IrhTNF for 120 min at 4°C in EMEM containing 0.5% gelatin as binding medium. The cells were then washed three times with chilled binding medium and lysed with 1 ml of 0.5 M NaOH, and 0.5 ml of the lysate was counted for radioactivity. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled rhTNF protein.

Quantification of Hydroxyl Radical Formation. Hydroxyl radical production was measured by the formation of methane from DMSO, in accordance with the method of Repine et al. (19). Each 2-ml reaction mixture was prepared in a siliconized 3.5-ml glass tube (Pierce, Rockford, IL) by the sequential addition of 1 ml of cell suspension (2 × 10⁷/ml), 0.4 ml of DMSO solution (2 M) and 0.4 ml of solution containing bipyridine, III 2F3 or mouse IgG as applicable, each of these in the culture medium described above but containing 20 mM HEPES (pH 7.2), followed by sealing of the tube with a silicon tellon septum and open-top screw cap, and injection of 0.2 ml of the same 20 mM HEPES medium containing rhTNF through the septum. The mixture was vigorously mixed and then incubated up to 24 h at 37°C in a shaking water bath; the reaction was terminated by placing the tube on melted ice.

A 0.2-ml sample of the headspace gas from each tube was withdrawn into a gas-tight syringe (Hamilton, Reno, NV), after its vigorous mixing by depression and withdrawal of the syringe plunger at least 10 times. The methane concentration in the 0.2-ml gas sample was determined on a Shimazu GC-9A gas chromatograph (Shimazu, Tokyo) equipped with a flame-ionization detector and a 3-mm x 2-m stainless steel column packed with 60/80 mesh active carbon (Gasukuro Kogyo, 1671

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Nitrogen gas was used as carrier, at a flow rate of 50 ml/min; injector, column, and detector temperatures were 200, 150, and 200°C, respectively. A calibration curve, linear over the range of 0.01–35 nmol methane, was constructed with a 99.7% methane standard (Gasukuro Kogyo). The retention time for authentic methane under these conditions was 1 min. The methane content, expressed as pmol of methane per 1.5 ml of headspace gas, was determined from the intersection on the calibration curve of the peak area after subtraction of the area corresponding to content of the laboratory air (14.2–18.2 pmol/0.2 ml). Because of the known tendency for variations to occur in the results of biological assays, each experiment was accompanied by an assay, by the method described above, for hydroxyl radical production by a control containing no rhTNF.

RESULTS

Suppression of rhTNF Cytotoxicity. The effects of various hydroxyl radical scavengers, iron chelators and antioxidant enzymes on rhTNF cytotoxicity, as determined for L-M cells, are shown in Table 1. In the presence of DMSO (400 mM) as hydroxyl radical scavenger and bipyridine (60 μM) as iron chelator and thus suppressor of hydroxyl radical formation (20), the cytotoxic effect of rhTNF was decreased to 24 and 63%, respectively, of that observed with rhTNF alone. In contrast, no significant reduction in cytotoxic effect was observed in the presence of the SOD or catalase antioxidant enzyme or the DETAPAC iron chelator, for all of which the cell membrane is impermeable (21–23). DETAPAC in fact apparently served to enhance the rhTNF cytotoxicity for reasons which are as yet unknown.

Time Course of Cytotoxic Activity of rhTNF on L-M Cells. Following incubations at 37°C for 0–24 h with or without rhTNF but in the absence of any hydroxyl radical scavenger, as shown in Fig. 1, cytotoxic assay by the dye uptake method showed no significant cell death at 0–15 h of incubation with rhTNF, but increasingly high levels of cell death at 18, 21, and 24 h of incubation periods.

Influence of DMSO Concentration on Methane Formation. The amount of methane produced in 18 h of L-M cell incubation after addition of DMSO at concentrations of 0–600 mM is shown in Fig. 2. In the absence of rhTNF, methane production reached a plateau at 100 mM DMSO, with no further increase at higher concentrations. Additional methane production apparently induced by the presence of rhTNF (100 U/ml), as represented by the shaded area in Fig. 2, reached a similar plateau at 400 mM DMSO, which was therefore the DMSO concentration utilized for all subsequent determinations of methane production.

<table>
<thead>
<tr>
<th>Agent Function</th>
<th>Concentration</th>
<th>rhTNF cytotoxicity (%)</th>
<th>Relative sensitivity to rhTNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>70.4 ± 1.87</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>SOD</td>
<td>66.3 ± 0.70</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>68.4 ± 0.93</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>SOD + catalase</td>
<td>66.0 ± 1.36</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>44.0 ± 1.24</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Bipyridine</td>
<td>44.0 ± 0.19</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>DETAPAC</td>
<td>86.5 ± 1.48</td>
<td>1.23</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated as cytotoxicity = 100 × (1 – OD of rhTNF treated cells/OD of rhTNF free cells).

** Calculated as relative sensitivity = rhTNF cytotoxicity with agent/rhTNF cytotoxicity without agent.

*** Mean ± SE of three separate experiments.

† Values significantly (*, P < 0.001; **, P < 0.005) different from value with no agent added.
HYDROXYL RADICAL PRODUCTION BY TNF

Fig. 1. Time course of cytotoxic activity of rhTNF on L-M cells. L-M cells (1 x 10^4 cells/200 μl) were added to wells of 96-well microculture plate, and incubated at 37°C for 4 h. Medium was aspirated and 200 μl EMEM containing rhTNF (100 U/ml) was added, followed by incubation at 37°C for the indicated periods in 5% CO₂. Cytotoxicity was assessed by dye uptake method as described previously. Percentage cytotoxicity was calculated by the formula shown in Table 1. Values are means ± SD of triplicate wells. *, **, values significantly (*, P < 0.005, **, P < 0.001) different from value without rhTNF.

INCUBATION TIME (hours)

Fig. 2. Effect of DMSO on the production of methane by L-M cells incubated with rhTNF. Each 3.5-ml tube contained 2 x 10^7 L-M cells and the indicated concentration of DMSO with (●) or without (○) 100 U/ml of rhTNF and was incubated at 37°C for 18 h. The concentration of methane in 200 nI of headspace gas was measured by gas chromatography and total produced methane was calculated as described in “Materials and Methods.” ● produced methane when medium containing only DMSO was incubated. Shaded area, produced methane induced by the presence of rhTNF. Values are mean ± SE of six separate preparations. *, **, *** values significantly (*, P < 0.05; **, P < 0.005; ***, P < 0.001) different from value without rhTNF.

DMSO CONCENTRATION (mM)

Fig. 3. Time course of the production methane by L-M cells incubated with rhTNF. L-M cells (2 x 10^7 cells/2 ml) were incubated at 37°C in medium containing 400 mM DMSO with (●) or without (○) the addition of rhTNF (100 U/ml). At indicated times, produced methane was assessed by gas chromatography as described in Fig. 1. No methane was detected when medium containing only 400 mM DMSO was incubated (●). Shaded area, produced methane induced by the presence of rhTNF. Values are means ± SE of eight separate preparations. *, **, values significantly (*, P < 0.05; **, P < 0.001) different from value without rhTNF.

INCUBATION TIME (hours)

Fig. 4. Production of methane by L-M cells as a function of rhTNF concentration. L-M cells (2 x 10^7 cells/2 ml) were indicated in medium containing 400 mM DMSO with indicated concentration of rhTNF at 37°C for 18 h. Produced methane was assessed by gas chromatography, as described in Fig. 1. Values are mean ± SE of eight separate preparations.

rhnTF CONCENTRATION (U/ml)

DISCUSSION

The hydroxyl radical is a highly potent reactive oxygen known to function as a direct mediator in the cytolytic effects of anticancer quinone (24), NK cells (25), neutrophils (26), and radiological exposure (27). TNF, on the other hand, reportedly stimulates the expulsion of superoxide anion by neutrophils (28), and hydrogen peroxide by macrophages (29). Its cytotoxic effect also reportedly is suppressed by hydroxyl radical scavengers (12–14), and by iron chelator, an inhibitor of hydroxyl radical production (13, 14), thus suggesting that hydroxyl radical production is involved in the mechanism of TNF cytotoxicity.

The present study provides the first quantitative evidence of a correspondence between hydroxyl radical production and TNF concentration, cell sensitivity, and cytotoxic effect, by the measurement of methane production from DMSO during incubation of three cell lines under various degrees of hydroxyl radical suppression and rhTNF concentration.

We first applied this method to investigation of the kinetics of hydroxyl radical production in incubation of TNF sensitive L-M cells. The results show that the increase in hydroxyl radical
The cells were then washed, followed by elution with 0.5 M NaOH and determination of the surface bound radioactivity. Values are mean ± SE of six separate preparations.

**Table 2 Effect of anti-rhTNF monoclonal antibody III 2F3 on cytotoxicity and cell surface binding of rhTNF**

Cytotoxicity was determined by the dye uptake method previously described. Total and nonspecific binding of $[^{125}I]$rhTNF were assessed as follows. L-M cells ($5 \times 10^5$ cells/500 µl binding medium) were incubated for 2 h at 4°C with $[^{125}I]$TNF (5 nM, 586 U/ml) in the presence or absence of unlabeled rhTNF (5 µM).

The cells were then washed, followed by elution with 0.5 M NaOH and determination of the surface bound radioactivity.

Table 2: Effect of anti-rhTNF monoclonal antibody III 2F3 on cytotoxicity and cell surface binding of rhTNF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytotoxicity*&lt;sup&gt;a&lt;/sup&gt; (mean ± SE, %)</th>
<th>Total binding of $[^{125}I]$rhTNF (mean ± SE, cpm)</th>
<th>Non-specific binding of $[^{125}I]$rhTNF (mean ± SE, cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>78.2 ± 2.5</td>
<td>2898 ± 103.2</td>
<td>266 ± 1.7</td>
</tr>
<tr>
<td>III 2F3*</td>
<td>1.2 ± 4.3</td>
<td>255 ± 5.3</td>
<td>252 ± 4.2</td>
</tr>
<tr>
<td>Mouse IgG*</td>
<td>76.5 ± 3.1</td>
<td>2686 ± 87.6</td>
<td>261 ± 3.5</td>
</tr>
</tbody>
</table>

* Mean ± SE of indicated number of experiments.

**Fig. 5.** Effect of iron chelator bipyridine on the production of methane by L- M cells incubated with rhTNF. L-M cells ($2 \times 10^5$ cells/2 ml) were incubated for 18 h at 37°C in medium containing 400 mM DMSO alone (a), with rhTNF at 100 U/ml (b), with rhTNF at 100 U/ml and III 2F3 at 10 µg/ml (c), or with rhTNF at 100 U/ml and mouse IgG at 10 µg/ml (d). L-M cell-free medium (2 ml) containing 400 mM DMSO and no rhTNF (e) or rhTNF (f) at 100 U/ml was incubated at 37°C for 18 h. Produced methane was assessed by gas chromatography as described in Fig. 1.

**Table 3 rhTNF-induced methane production by KYM and HEL cells**

Cytotoxicity was determined by the dye uptake method previously described. Methane production per $2 \times 10^5$ cells in 18 h was assessed by gas chromatography as described in Fig. 1.

<table>
<thead>
<tr>
<th>Cell</th>
<th>rhTNF (U/ml)</th>
<th>Cytotoxicity&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Number of experiments</th>
<th>Produced methane (pmol/2 × 10&lt;sup&gt;5&lt;/sup&gt; cells/18 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-M</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>22.0 ± 4.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>70.4</td>
<td>8</td>
<td>48.0 ± 3.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>KYM</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>15.7 ± 4.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>76.0</td>
<td>8</td>
<td>39.3 ± 5.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HEL</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>13.2 ± 3.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
<td></td>
<td>13.3 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Calculated from the formula shown in Table 1.

<sup>a</sup> Mean ± SE of indicated number of experiments.

<sup>b</sup> NS, not significant.

**Fig. 6.** Effect of anti-rhTNF monoclonal antibody III 2F3 on the production of methane by L-M cells incubated with rhTNF. L-M cells ($2 \times 10^5$ cells/2 ml) were incubated at 37°C for 18 h in medium containing 400 mM DMSO alone (a), with rhTNF at 100 U/ml (b), with rhTNF at 100 U/ml and III 2F3 at 10 µg/ml (c), or with rhTNF at 100 U/ml and mouse IgG at 10 µg/ml (d). L-M cell-free medium (2 ml) containing 400 mM DMSO and no rhTNF (e) or rhTNF (f) at 100 U/ml was incubated at 37°C for 18 h. Produced methane was assessed by gas chromatography as described in Fig. 1.

The correlation between TNF cytotoxic effect and hydroxyl radical production was further shown by the L-M cell incubation in the presence of rhTNF and bipyridine. In the presence of bipyridine, with the resulting suppression of rhTNF-stimulated hydroxyl radical production, the cytotoxic effect of rhTNF on the L-M cells was inhibited significantly, suggesting that the stimulation of hydroxyl radical production is an important part of the mechanism of rhTNF cytotoxicity.

Comparison of the observed time course of rhTNF cytotoxicity in the absence of hydroxyl radical scavenger with that of the hydroxyl radical production induced by rhTNF indicates that the cytotoxic effect and the increased hydroxyl radical production are approximately concurrent. The observed inhibition of rhTNF cytotoxicity by DMSO and bipyridine, furthermore, suggests that hydroxyl radical production is rather a cause than a result of cell death.

The investigation with KYM and HEL cell lines also showed a correlation between rhTNF sensitivity and hydroxyl radical production. In incubation of TNF-sensitive KYM cells, the addition of rhTNF resulted in an increase in hydroxyl radical production effected by the presence of rhTNF, in terms of the difference between its production with and without rhTNF, is dependent on both incubation time and rhTNF concentration.

The substantial hydroxyl radical production by cells in the absence of rhTNF, as observed in this and the subsequent experiments of the present study, may be attributed to mitochondrial respiration and the other redox known to occur in cells and thus generate hydroxyl radicals, and is in accord with similar findings by other investigators for phagocytes and Erlich tumor cells in vitro in the presence of DMSO alone (19, 24).

The next series of experiments were performed to determine whether TNF is directly involved in the reaction yielding hydroxyl radical in the manner of anticancer quinone and menadione, which are known to function as electron acceptors and thus produce hydroxyl radicals by reduction of oxygen molecules (24, 30). The results tended to preclude this possibility, and show rather that TNF promotes hydroxyl radical production through stimulation of intracellular production, as: (a) no suppression of rhTNF cytotoxicity occurred in L-M cell incubations with extracellular reactive oxygen suppressed by SOD, catalase, or DETAPAC; (b) no observable increase of hydroxyl radical production during incubation of cell-free medium was effected by the addition of rhTNF; and (c) the rhTNF stimulation of hydroxyl radical production was apparently suppressed completely by the addition of the monoclonal antibody III 2F3, which was shown to block rhTNF receptor binding and completely neutralize rhTNF cytotoxicity.
production levels similar to that found for L-M cells. In the incubation of TNF-insensitive HEL cells under the same conditions, however, no observable increase in hydroxyl radical production resulted from the addition of rhTNF.

The results of this study thus clearly indicate that rhTNF stimulates production of the hydroxyl radical by rhTNF-sensitive cells, and that this radical plays an important role in the overall mechanism of rhTNF cytotoxicity. It is not yet possible to provide a definitive description of the mechanism of the stimulated production or the precise role of the radical in the overall mechanism of rhTNF cytotoxicity, but a partial explanation may be attempted in the light of the present study and related findings.

Various recent reports have shown that TNF cytotoxicity is effectively suppressed by a mitochondrial inhibitor (11, 12) and by inhibitors of arachidonic acid metabolism (13, 14). Moreover, both the mitochondrial electron-transfer chain (31) and the arachidonic acid cascade (32) are known to generate the hydroxyl radical. It is therefore possible that the rhTNF-stimulated increase in hydroxyl radical production as observed in the present study may be caused by activation of either or both of these metabolic systems.

The hydroxyl radical, on the other hand, is known to induce lipid peroxidation (33) and to cause activation of lysosomal enzyme by its induction of lipid peroxidation in lysosomal membrane (34), and also to directly effect DNA chain fragmentation (35). Lipid peroxidation (13), lysosomal enzyme activation (12), and DNA fragmentation (36) have all been reported to occur in cells under stimulation by TNF. It would thus appear reasonable to suggest that the hydroxyl radical induced by TNF could be the actual mediator of these intracellular reactions.

ACKNOWLEDGMENTS

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33. Sugioka, K., Nakano, M., Tero-Kubota, S., and Ikegami, Y. Generation of hydroxyl radical by its induction of lipid peroxidation in lysosomal enzyme by its induction of lipid peroxidation in lysosomal membrane (34), and also to directly effect DNA chain fragmentation (35). Lipid peroxidation (13), lysosomal enzyme activation (12), and DNA fragmentation (36) have all been reported to occur in cells under stimulation by TNF. It would thus appear reasonable to suggest that the hydroxyl radical induced by TNF could be the actual mediator of these intracellular reactions.

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