Maintenance and Cure of the L5178Y Murine Tumor-dormant State by Interleukin 2: In Vivo and in Vitro Effects

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ABSTRACT

We studied the antitumor effects of recombinant human interleukin 2 (HuIL-2) in DBA/2 mice which harbor L5178Y lymphoma cells in a tumor-dormant state in the peritoneal cavity and in peritoneal cell (PC) cultures prepared from such mice. Intraperitoneal injection of 10,000 to 100,000 units of HuIL-2/day for 10 days eliminated tumor cells from 30–45% of the mice, whereas no mice became tumor-free in the phosphate-buffered saline-treated control group. In vitro, as little as 10 units/well HuIL-2 stimulated antitumor cytotoxic activity in PC from tumor-dormant mice, whereas HuIL-2 in concentrations up to 1,000 units/well HuIL-2 failed to stimulate cytotoxic activity in PC from normal mice. HuIL-2 directly stimulated antitumor cytotoxic activity in nonadherent but not in adherent PC cultures from tumor-dormant mice; however, treatment of whole PC from tumor-dormant mice with HuIL-2 resulted in the development of antitumor cytotoxic activity in the adherent PC population which were derived from such cultures. This suggests that the HuIL-2–treated nonadherent PC contributed to the cytotoxic activation of the adherent PC. Flow cytometric analysis of the PC from tumor-dormant mice revealed a polyclonal expansion of T-lymphocytes. Lyt-1+, Lyt-2+, and L3T4+ lymphocytes were all required for HuIL-2 to induce antitumor cytotoxic activity.

INTRODUCTION

We have reported that a tumor-dormant state can be established in DBA/2 by s.c. immunization and i.p. challenge with a weakly metastatic line of the methylcholanthrene-induced syngeneic T-cell lymphoma L5178Y. This tumor-dormant state is maintained by immunological mechanisms (1, 2) involving CTL3 and macrophages which can act either separately or in synergy (3–5). The tumor-dormant state can be subverted with rapid formation of ascitic tumors by treatment of mice with prostaglandin E2 and enhanced with elimination of tumor cells by treatment with indomethacin, an inhibitor of endogenous prostaglandin E2 production (6). We have studied the mechanisms by which the progressive growth of L5178Y cells is restrained in the peritoneal cavity of mice which harbor them in a tumor-dormant state. Peritoneal cell cultures prepared from these mice contain small numbers of the ‘dormant’ L5178Y cells and the progressive growth of these tumor cells is restrained in most of the cultures (6). These PC produce small amounts of endogenous interferon-gamma (MuINF-γ) and murine tumor necrosis factor (MuTNF), and treatment of these cultures with antibodies to either of these cytokines results in progressive growth of the tumor cells (7). Murine INF-γ requires and synergizes with murine tumor necrosis factor to induce its antitumor effects (7–9).

Interleukin 2, first described as a T-cell growth factor (10), has been shown to stimulate the growth and enhance the functional capabilities of activated T-lymphocytes. Interleukin 2 supports the growth of CTL (11, 12) and NK cells (13), augments NK cell function (14, 15), and is the essential factor required for the induction and growth of LAK cells (16). Interleukin 2 induces INF-γ in human peripheral blood monocytes and requires INF-γ to induce activated killer cells and a proliferative response (17). Interleukin 2 has also been shown to produce antitumor effects in vivo against a variety of human and animal tumors, when administered either alone or in conjunction with LAK cells (18–22).

We evaluated the therapeutic effects of HuIL-2 in L5178Y tumor-dormant mice because of its demonstrated ability to expand the population of cytotoxic effector cells in vivo and in vitro (22–24). We previously showed that cytotoxic activity in peritoneal T-lymphocytes reached maximum levels 4 days after i.p. L5178Y cell challenge of immunized DBA/2 mice and then rapidly declined, reaching background levels after 40–50 days (1). The objectives of the study presented here were: (a) to determine whether IL-2 could cure mice which harbor L5178Y cells in a tumor-dormant state and (b) to study the mechanisms by which an antitumor effect was produced. We found that treatment of tumor-dormant mice in vivo with high doses of HuIL-2 eliminated tumor cells from 30 to 45% of the mice, whereas no mice became tumor-free in the PBS-treated control group. In vitro experiments with PC cultures from tumor-dormant mice revealed that the antitumor activity of HuIL-2 was mediated by both the NAD-PC and AD-PC populations.

MATERIALS AND METHODS

Animals. Eight to 12-week-old female DBA/2 mice were obtained from The Jackson Memorial Laboratories (Bar Harbor, ME). They were housed in a temperature-controlled room with a cycle of 12 h of light and 12 h of dark.

Cells. The L5178Y cell line is a nonmetastatic T-cell lymphoma induced in DBA/2 mice with methylcholanthrene (1) and passed in vivo and in vitro. YAC-1 is an A strain Moloney virus-induced lymphoma, EL-4 is a benzanthracine-induced mouse lymphoma, and FLC-745 is a Friend virus-induced erythroleukemic cell.

Culture Medium. MEM was supplemented with endotoxin-free 10% fetal bovine serum, sodium pyruvate (1 mm), nonessential amino acids (0.1 mm), l-glutamine (2 mm), 15 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, sodium bicarbonate (1.125 g/liter), and 50 µg/ml gentamycin sulfate (Schering Corporation).

Reagents. HuIL-2 was supplied by Hoffmann-La Roche (Nutley, NJ). The units of HuIL-2 were determined by CTL cell proliferation...
Each cell suspension was titrated in quadruplicate. The plates were incubated at 37°C in a humidified 5% CO2 atmosphere and examined by the SEPD assay (11, 12). The HuIL-2 was supplemented with 1% heat-inactivated normal mouse serum, as was the PBS used in experimental control groups. The mouse serum contained <0.003 ng/ml endotoxin, as measured in a Limulus amoebocyte assay. The culture supernatants were derived from rat anti-mouse hybridoma GK1.5 provided by Dr. F. Fitch of University of Chicago. The antibody reacts with the L3T4 (CD4) molecules and has been shown to be of the IgG2b subclass (25). The culture supernatants from rat anti-mouse hybridoma 3.168 were provided by Dr. R. Kornfeld of Thomas Jefferson University. This antibody reacts with the Lyt-2 (CD8) molecules and has been shown to be of the IgM class (26). Monoclonal antibody to Lyt-1 was purchased from Becton-Dickinson. FITC-conjugated anti-Thy-1.2, Lyt-1, Lyt-2, and L3T4 monoclonal antibodies were purchased from Becton-Dickinson. All reagents contained <0.003 ng/ml endotoxin/ml in their final working concentrations, as measured by the Limulus amoebocyte lysate assay.

Establishment of the L5178Y Tumor-dormant State. The protocol to establish the L5178Y tumor-dormant state in DBA/2 mice has been described (1). Briefly, 1 x 10^6 in vivo-passaged L5178Y cells were implanted s.c. on the mid-ventral surface of DBA/2 mice. Seven to 10 days later, the resultant 0.5- to 1.0-cm diameter nodules were surgically excised, and 7 days later the mice were challenged i.p. with 5 x 10^4 L5178Y cells.

PPL and CPL. For the PPL, mice received an i.p. injection of 2.5 ml of sterile pyrogen-free PBS and the peritoneum was massaged thoroughly to mix the PBS with the peritoneal contents. The mice were then lightly anesthetized with ether and restrained on a board, ventral side upward. A small area of the ventral surface was shaved and 0.4 to 2 ml of the PBS was removed from the peritoneal cavity with a 5-ml syringe with a 25-gauge needle. The volume removed from each sampling was recorded. The recovered PC were pelleted and resuspended in the same volume of MEM. For the CPL, mice were killed by cervical dislocation and the PC were removed in two successive 5-ml peritoneal washouts with PBS. The PC were pelleted, resuspended in 4 ml MEM, and counted by hemocytometer. This technique recovered >99% of the PC (6) and provides cell samples that are quantitatively representative of the entire peritoneal cell contents (27).

SEPD Assay for Enumeration of Tumor Cells. Single-cell suspensions of tumor cell populations were prepared at a known volume in MEM. One hundred µl of MEM were added to each well of a 96-well flat-bottomed microtiter plate (Costar, Cambridge, MA). Ten thousand L5178Y lymphoma cells in log phase growth were added to each well, including a L5178Y cell alone group as a control, and the volume in each well was brought to 0.3 ml. The plates were incubated for 7 days at 37°C and tumor cell growth in each well was evaluated microscopically. The cells in the wells were then suspended and the tumor cells were quantitated by the SEPD assay and compared with the number of tumor cells calculated to be in the wells at the start of culture.

Separation of NAD-PC and AD-PC. The techniques involving adherence to plastic have been described (7).

Assay for Macrophage Cytotoxic Activity. Peritoneal exudate cells were obtained from tumor-dormant mice and were suspended in MEM with 5% fetal calf serum. Four hundred thousand PC from tumor-dormant mice with or without HuIL-2 were plated in 0.3 ml into wells of a 96-well flat-bottomed microtiter plate (Costar). After a 48-h incubation at 37°C in a CO2 incubator, the NAD-PC (containing the L5178Y cells) were removed by repeated washing (3 times) with warm PBS. Ten thousand L5178Y lymphoma cells in log phase growth were added to each well, including a L5178Y cell alone group as a control, and the final volume was brought to 0.2 ml. After a 40 h incubation at 37°C, 0.5 µCi of [3H]TdR (NEN DuPont) was added to each well for an 8-h pulse. Labeled cells were collected on a glass filter, and the radioactivity trapped in the filter was counted in a liquid scintillation counter. Results are expressed as the percentage of cytotoxicity according to the following formula.

% Cytotoxicity = \frac{cpm \text{ in culture containing macrophages} - cpm \text{ in cultures not containing macrophages}}{cpm \text{ in cultures not containing macrophages}} \times 100

Antibody-dependent Complement-mediated Cytolysis. The PC were incubated with antibodies to Lyt-1, Lyt-2, or L3T4 for 30 min at room temperature and washed 3 times. They were then incubated further with rabbit complement (Pel-Freeze, WI) for 45 min at 37°C and washed 3 times.

Quantitation of Lymphocyte Subsets in PC by Flow Cytometry. The PC samples from mice obtained by PPL were washed with PBS-BSA-azide solution (0.1 M PBS plus 1% BSA plus 0.1% sodium azide) 3 times. The total number of PC were counted with a Coulter counter. The PC concentration was adjusted to 2 x 10^6 cells/0.1 ml/well and stained with the FITC-conjugated monoclonal antibodies for 30 min

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Fig. 1. Protocol for in vivo treatment of the tumor-dormant state by HuIL-2. L5178Y cell-immunized and challenged mice were subjected to a PPL on the 25th day post challenge. The tumor burden was quantitated by the SEPD assay, and mice were classified as tumor-dormant if they had between 1 and 1 x 10^4 tumor cells in their peritoneal cavity. Five days after PPL, HuIL-2 or PBS was administered for 10 days and, 10 days after the last inoculation, CPLs were performed and all cells were placed in culture and quantitated, as described in Materials and Methods. Tumor burdens before and after treatment in individual mice were then compared.
**Therapeutic Effects of IL-2 on Tumor-Dormant State**

Table 1: Antitumor effect of HuIL-2 in mice harboring L5178Y cells in a tumor-dormant state

<table>
<thead>
<tr>
<th>Mouse</th>
<th>PBS</th>
<th>1,000 units IL-2/day</th>
<th>10,000 units IL-2/day</th>
<th>100,000 units IL-2/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>1</td>
<td>480</td>
<td>Ascites (Et)</td>
<td>614</td>
<td>Ascites (Et)</td>
</tr>
<tr>
<td>2</td>
<td>108</td>
<td>Ascites (Et)</td>
<td>64</td>
<td>87 (D)</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>Ascites (Et)</td>
<td>16</td>
<td>99,229 (Et)</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>24,803 (Et)</td>
<td>5</td>
<td>3 (D)</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>Ascites (Et)</td>
<td>2</td>
<td>4,915 (Et)</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>983 (Et)</td>
<td>1</td>
<td>17 (D)</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>Ascites (Et)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>123 (D)</td>
<td>1</td>
<td>92 (D)</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>1 (D)</td>
<td>1</td>
<td>23,593 (Et)</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>Ascites (Et)</td>
<td>1</td>
<td>0 (C)</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>1 (D)</td>
<td>1</td>
<td>22.2</td>
</tr>
</tbody>
</table>

*Ascites rate is expressed as the number of mice which developed ascites initiation of treatment over the total number of mice × 100.

*Cure rate is expressed as the number of mice with no recoverable tumor cells in their peritoneal cavity over the total number of mice × 100.

*Decrease in tumor burden rate is expressed as the number of mice with 4-fold fewer tumor cells in their peritoneal cavity at the end of treatment compared with the number of tumor cells before treatment over the total number of mice × 100.

**Effect of HuIL-2 on the L5178Y Tumor-Dormant State.** The effects of i.p. inoculations of graded doses of HuIL-2 in mice harboring L5178Y cells in a tumor-dormant state were studied. Interleukin 2 was diluted in PBS to 500, 5,000, and 50,000 units/0.2 ml and administered twice a day for 10 days, with one additional group receiving an appropriate PBS-diluted control preparation. Using the protocol described in Fig. 1, 11 tumor-dormant mice were treated with PBS, 9 mice with HuIL-2 at 1,000 units/day, 10 mice with HuIL-2 at 10,000 units/day, and 11 mice with HuIL-2 at 100,000 units/day.

No toxicity was observed during the entire experimental period, but at the time of sacrifice there was a significant reduction in weight in all of the HuIL-2-treated groups, especially in the high-dose group, as compared with the PBS-treated group. As seen in Table 1, treatment of DBA/2 mice which harbor L5178Y lymphoma cells in a tumor-dormant state with HuIL-2 at 10,000 to 100,000 units/day for 10 days i.p. decreased the tumor burden and eliminated tumor cells from 30 to 45% of the mice, whereas only 9% of mice in the PBS-treated control group had a decrease in tumor cell numbers and no mice became tumor-free. Similar results were obtained in two separate experiments.

**Effect of HuIL-2 on Tumor Cell Growth in PC Cultures from Normal and Tumor-Dormant Mice**. Peritoneal cells from pools of two normal and of two tumor-dormant mice were planted in wells of a 96-well flat-bottomed microtiter plate at 4 × 10³ cells/well. Two thousand in vivo passage L5178Y cells were added to each of the wells which contained normal PC. The number of tumor cells in the PC from the tumor-dormant mouse pool was determined by the SEPD assay. Under these culture conditions, the tumor cells in many of the PC cultures from tumor-dormant mice and in all of the PC from normal mice proliferate progressively. Interleukin 2 was added at 1, 10, 100, and 1000 units/0.3 ml/well and MEM was added to the wells of the control group, with each group containing three wells. After 7 days of incubation, the cells in all of the culture wells were suspended and the number of tumor cells in each well was quantitated by the SEPD assay.

**Figure 2** shows that HuIL-2 at all concentrations failed to inhibit tumor cell growth in normal PC cultures. In contrast, as little as 10 units/0.3 ml/well of HuIL-2 produced a significant antitumor effect in PC cultures from tumor-dormant mice. In three experiments, HuIL-2 promoted elimination of L5178Y cells from PC cultures from tumor-dormant mice. In control experiments (data not shown), HuIL-2 at doses up to 10,000 units/0.3ml/well produced no direct inhibitory effect on the proliferation of pure cultures of L5178Y cells.

**Kinetics of Tumor Cell Killing by HuIL-2 in PC Cultures from Tumor-Dormant Mice**. We next determined the kinetics of tumor cell killing by HuIL-2 in PC cultures from tumor-dormant mice. Fig. 3 shows the results of an experiment in which culture wells containing PC from a pool of three tumor-dormant mice were treated with MEM control and 100 units/0.3 ml/well HuIL-2 and the tumor cells in the three wells in each group were quantitated on days 3, 5, and 7 after treatment. Tumor cells grew progressively in the MEM-treated control wells, whereas the number of tumor cells in the HuIL-2-treated wells decreased on days 3 and 5, with little additional decrease between days 5 and 7.

**Stimulation of Cytotoxic Activity in the NAD-PC from Tumor-Dormant Mice by HuIL-2**. We next determined whether HuIL-2 stimulated cytotoxic activity in the NAD-PC from tumor-dormant mice. NAD-PC cultures were prepared from individual mice in wells of a 96-well microtiter plate, as described in "Materials and Methods" and sets (3 wells/set) were treated with either MEM or HuIL-2 at 100 units/0.3 ml/well. The
was designed to identify the lymphocyte populations in PC cultures from tumor-dormant mice which are required for HuIL-2 to induce its antitumor effects. We sought to do this with PC populations from which the Lyt-1-, Lyt-2-, and L3T4-expressing lymphocytes had been eliminated using cytotoxic treatment with monoclonal antibodies to Lyt-1, Lyt-2, and L3T4 in the presence of rabbit complement. Before these experiments could be conducted, it was necessary to determine whether the antibodies would affect the L5178Y cells which are associated with the PC from tumor-dormant mice. We therefore evaluated the expression of Lyt-1, Lyt-2, and L3T4 antigens on L5178Y cells by treating these cells with specific monoclonal antibodies and complement and quantitating residual cells by dye exclusion; the cytotoxic effects of these antibodies on normal thymus and spleen served as controls. We found that Lyt-1, Lyt-2, and L3T4 antigens were not expressed on L5178Y cells (data not shown).

PC from tumor-dormant mice were then treated with antibodies to Lyt-1, Lyt-2, and L3T4 in the presence of complement. Treated and untreated cells were then cultured with or without HuIL-2 or MEM at 37°C. After 7 days of incubation, the number of tumor cells in the culture wells was determined by the SEPD assay. As seen in Fig. 4, HuIL-2 alone produced a very marked antitumor effect as compared with MEM. Elimination of any one of the Lyt-1-, Lyt-2-, or L3T4-expressing lymphocyte populations prior to treatment with HuIL-2 prevented HuIL-2 from producing its antitumor effect. Similar results were obtained in another experiment.

Quantitation of Lymphocyte Subsets in the PC from Normal and Tumor-dormant Mice by Flow Cytometry. We next quantitated lymphocyte subsets in normal and tumor-dormant mice using flow cytometric analysis. The PC from 8 normal mice and from 10 tumor-dormant mice were removed by CPL and PPL, respectively, and washed with PBS-BSA-azide 3 times and the PC were quantitated in a Coulter counter. The cell concentrations were adjusted to $2 \times 10^6/0.1$ ml/well. The PC were stained with FITC-conjugated monoclonal antibodies for 30 min on ice and, after washing with PBS-BSA-azide buffer, were analyzed in an EPICS V flow cytometer. As seen in Table 3, in $1 \times 10^6$ cells analyzed, the normal PC population contained about $1.39 \times 10^5$ Lyt-1-, $0.86 \times 10^5$ Lyt-2-, and $1.20 \times 10^5$ L3T4-positive cells. In low tumor burden tumor-dormant mice, the number of all T-cell subsets was not different from the normal but, in the high tumor burden tumor-dormant mice, the number of all T-cell subsets was increased and the percentage of lymphocytes was also increased. The differences between the total number of Lyt-1-, Lyt-2-, and L3T4-expressing lym-
In Table 4, Experiment 1, the NAD-PC were removed after a 2-h incubation and sets of wells containing the AD-PC (4 wells/set) were incubated with MEM and with HuIL-2 at 100 units/0.3 ml/well for 48 h. The cultures were then washed and 1000 L5178Y cells were added to each well. The plates were incubated further for 7 days and the number of tumor cells per well was quantitated by the SEPD assay. In Experiment 2, the whole PC were treated with either MEM or HuIL-2 at 100 units/0.3 ml/well and, after a 48-h incubation, the NAD-PC were removed. One thousand L5178Y cells were then added to each well, the cultures were incubated further for 7 days, and the number of tumor cells per well was quantitated by the SEPD assay. As seen in Experiment 1, no antitumor activity was detected in the AD-PC which were treated with HuIL-2 at the onset of culture. However, the AD-PC which were derived from HuIL-2-treated whole PC cultures from tumor-dormant but not normal mice developed marked antitumor activity (Experiment 2). This suggests that the NAD-PC in HuIL-2-treated whole PC cultures contribute to the development of antitumor activity in the AD-PC population.

In Table 5, the basic protocol used in Table 4 was followed but after a 48-h incubation of the cultures with MEM and HuIL-2, the cultures were then washed and 10,000 L5178Y cells were added to each AD-PC-containing well. The plates were incubated further for 40 h, 0.5 μCi of [3H]Tdr was then added to each well for a 8-h pulse, and the incorporated 3H was quantitated in a liquid scintillation counter. As seen in Table 5 (Experiment 1), no cytotoxic activity was detected in the AD-PC which were treated with HuIL-2 at the onset of culture. However, the AD-PC which were derived from HuIL-2-treated whole PC cultures from tumor-dormant but not normal mice developed marked cytotoxic activity (Experiment 2). This suggests that the NAD-PC in HuIL-2-treated whole PC cultures contribute to the cytotoxic activation of the macrophage population.

**DISCUSSION**

We have used a unique animal model of tumor dormancy to evaluate the possible therapeutic effects of HuIL-2. In vivo, we found that high doses of HuIL-2 (10,000 to 100,000 units/day) could decrease the number of tumor cells and cure 30–45% of tumor-dormant mice, whereas only 9% of the mice in the PBS-treated control group had a decrease in tumor cell numbers and no mice became tumor-free. In the 25-day period of the HuIL-2 treatment experiment, 55% of the PBS-treated control mice developed ascitic tumors, whereas only 0–20% of the HuIL-2-treated mice developed tumors, suggesting that IL-2 may be important in the maintenance of the tumor-dormant state.
antitumor activity. Both L3T4 and Lyt-2 lymphocytes have been shown to produce MuIFN-γ (29). We found that depletion of any of the Lyt-1-, Lyt-2-, or L3T4-expressing lymphocyte populations from PC cultures results in decrease of MuIFN-γ production by HuIL-2 (30). This suggests that the tumor growth-inhibiting effect of HuIL-2 involves the production of MuIFN-α by the cooperative interactions of Lyt-1-, Lyt-2-, and L3T4-expressing lymphocytes. IFN-γ could have induced cytotoxic activity in lymphocytes and macrophages and augmented NK cell activity. We found that HuIL-2 treatment of the NAD-PC but not the AD-PC population stimulated antitumor activity. However, HuIL-2 treatment of whole PC resulted in the induction of both antitumor (Table 4) and cytotoxic (Table 5) activity in the AD-PC population derived from these cultures. In the accompanying paper on the LS178Y tumor-dormant system (30), we will report that HuIL-2 induces MuIFN-γ in PC from tumor-dormant mice but not from normal mice, that this MuIFN-γ is required and synergizes with IL-2 to stimulate antitumor activity in the lymphocyte population, and that the induced MuIFN-γ itself activates macrophages to a cytotoxic state.

We found that mice harboring LS178Y cells in a tumor-dormant state underwent a pan-T-lymphocyte expansion (Table 3). The ability of HuIL-2 to stimulate antitumor activity in the NAD-PC population suggests that IL-2 either stimulated CTL proliferation directly or indirectly by stimulating helper T-lymphocytes to produce more IL-2 receptors, as has been reported elsewhere (31, 32). This indirect feedback loop could have induced large scale CTL clonal expansion which could have lysed the tumor cells. In other systems the antitumor activity induced by IL-2 has been found to be mediated by cytotoxic T lymphocytes, nonspecifically activated lymphocytes (LAK cells), and NK cells (13-15). Cytotoxic T lymphocytes have been shown to be generated during the tumor-dormant state (3) and may have played a major role in the antitumor effects induced by HuIL-2 which are reported here.

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