Chronic Lymphocytic Leukemia B Cells Are Resistant to the Apoptotic Effects of Transforming Growth Factor-β

By Raymond S. Douglas, Renold J. Capocasale, Roberta J. Lamb, Peter C. Nowell, and Jonni S. Moore

Chronic lymphocytic leukemia (CLL) is the most common leukemia of the western world and is characterized by a slowly progressing accumulation of clonal CD5⁺ B cells. Our laboratory has investigated the role of transforming growth factor-β (TGF-β) and interleukin-4 (IL-4) in the pathogenesis of B-cell expansion in CLL. In vitro addition of TGF-β did not increase spontaneous apoptosis of B cells from most CLL patients, as determined using the TUNEL method, compared with a twofold increase observed in cultures of normal B cells. There was similar expression of TGF-β type II receptors on both CLL B cells and normal B cells. In contrast to apoptosis, CLL B-cell proliferation was variably inhibited with addition of TGF-β. In vitro addition of IL-4, previously reported to promote CLL B-cell survival, dramatically reduced spontaneous apoptosis of CLL B cells compared with normal B cells. CLL B-cell expression of IL-4 receptors was increased compared to normal B cells. Thus, our results show aberrant apoptotic responses of CLL B cells to TGF-β and IL-4, perhaps contributing to the relative expansion of the neoplastic clone.

MATERIALS AND METHODS

Patients, cell isolation, and incubation criteria. Peripheral blood mononuclear cells (PBMC) were obtained from 20 B-CLL patients being followed cytogenetically and immunologically in our laboratory. These patients had a clinical Rai stage of 0 to 4, and none were receiving chemotherapy when studied. PBMC were isolated from the peripheral blood (PB) of patients by density gradient centrifugation of heparinized blood on Ficoll-Hypaque (Pharmaca, Piscataway, NJ). B-cell-enriched fractions were obtained by rosetting with neuraminidase-treated sheep red blood cells (SRBC) and centrifugation on Ficoll-Hypaque. The resulting E-rosette-negative population contained greater than 98% CD19⁺, CD5⁺ B cells with no detectable T cells, and less than 2% monocytes. Purified B cells were isolated from the PB of normal donors by positive selection using a CD19 column (CellPro, Bothell, WA). The enriched population was greater than 95% B cells (CD19⁺ and CD20⁻) as determined by flow cytometry.

For all assays except TGF-β production, B lymphocytes were cultured at a final concentration of 1 × 10⁶ cells/mL in Optimem media (GIBCO-BRL, Grand Island, NY) supplemented with 5% fetal calf serum (FCS) and 2 mmol/L glutamine. For an enzyme-linked immunosorbent assay (ELISA) for TGF-β production, B lymphocytes were cultured in Optimem media supplemented with 1% FCS and 2 mmol/L glutamine. TGF-β1 (R&D), IL-4 (10 ng/mL; R&D, Minneapolis, MN), Staphylococcus aureus, and IL-2 (10 ⁶ cells/mL) were added in various combinations at culture initiation.

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quantitated using propidium iodide (Sigma) exclusion. Briefly, cells were cultured at uniform density for various times up to 10 days. Aliquots of 400 μL were resuspended and harvested in triplicate, and 100 μL of propidium iodide (10 μg/mL) was added 1 minute before analysis by flow cytometry. All events were acquired for 60 seconds at a constant flow rate using a FACScan flow cytometer (Becton Dickinson).

Quantitation of active and latent TGF-β1 production by ELISA. Supernatants were collected from CLL B cells or normal CD19+ B cells and quantitation of biologically active TGF-β1 was accomplished using the Transforming Growth Factor-β-1 ELISA System (Promega, Madison, WI). To determine the amount of naturally activated TGF-β1, samples were processed directly after harvest.

Quantitation of apoptosis by flow cytometry. Apoptosis was measured using the TUNEL (Tdt-mediated dUTP-FITC nick end labeling) assay, with minor modification from Gorczyca et al.23,24 Briefly, approximately 10^6 cells were harvested, washed twice, and fixed with 1% paraformaldehyde for 15 minutes. After washing, the cells were permeabilized with 0.1% Triton (Sigma) for 5 minutes and then washed twice. The labeling reaction was performed in a heating block for 1 hour at 37°C with 0.3 nmol FITC-12-dUTP (Boehringer Mannheim, Indianapolis, IN), 3 nmol dATP, 2.5 mmol/L CoCl₂, 12.5 U Tdt, and 5 mL of 5× Tdt Buffer (Boehringer Mannheim) in a total volume of 50 μL. Cells were then washed twice and analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) equipped with a 15-mW air-cooled 488-nm argon laser. Gating to exclude debris was based on diminished forward scatter (FSC) and side scatter (SSC). A minimum of 10,000 events was collected per sample and all analyses were performed with LYSIS II or CELLQuest software (BDIS).

Quantitation of viable cell number. Viable cell number was quantitated using propidium iodide (Sigma) exclusion. Briefly, cells were cultured at uniform density for various times up to 10 days. Aliquots of 400 μL were resuspended and harvested in triplicate, and 100 μL of propidium iodide (10 μg/mL) was added 1 minute before analysis by flow cytometry. All events were acquired for 60 seconds at a constant flow rate using a FACScan flow cytometer (Becton Dickinson).

Fig 1. TGF-β increases spontaneous apoptosis of normal peripheral blood B cells after 48 hours in culture. Apoptosis was determined using the TUNEL assay. Representative result from one of seven experiments. (A) Spontaneous apoptosis of normal B cells after 48 hours in culture (control). (B) Apoptosis of B cells with addition of 5 ng/mL TGF-β for 48 hours.

Fig 2. CLL B cells are resistant to TGF-β--mediated apoptosis. (A) CLL B cells from 13 patients or normal B cells (n = 7) were cultured alone (■) or with 5 ng/mL TGF-β (■) for 48 hours. Apoptosis was assayed by TUNEL, and the percentage of apoptotic cells is shown for each patient and for normals. (B) Percent change in spontaneous apoptosis upon addition of TGF-β is calculated as [(percent apoptosis with TGF-β/spontaneous apoptosis) × 100%] (P < .002).
DEFECTIVE TGF/β REGULATION OF B-CLL

without further treatment. To assay for total TGF-β1, samples were acid-activated by treatment with 1N HCl for 15 minutes at room temperature and then neutralized by the addition of 1N NaOH. The sensitivity of this system was in the range of 15 to 1,000 pg/mL.

Expression of TGF-β receptor type II and IL-4 receptor. Surface staining of TGF-β receptor type II was performed as recently described. To reduce nonspecific antibody binding, unlabeled rat IgG (20 μg/10⁶ cells; Sigma) and normal goat serum (20 μg/10⁶ cells; Sigma) were added for 5 minutes on ice. Polyclonal rabbit anti-human TGF-β receptor type II (6.1 μg/10⁶ cells; Upstate Biotechnology, Lake Placid, NY) was added for 30 minutes and the cells were subsequently washed twice. Fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (100 μg/10⁶ cells; Sigma) and normal goat serum (20 μg/10⁶ cells) were then added for 30 minutes. After the cells were washed twice, anti-human CD19-PE (Immunotech, Marseille, France) was added, and the cells were analyzed with a FACScan flow cytometer (Becton Dickinson).

Expression of the IL-4 receptor was also assessed using flow cytometry. Briefly, approximately 10⁶ B cells were harvested, washed, and incubated with rat IgG (Sigma; 10 μg/10⁶ cells) for 10 minutes to reduce nonspecific antibody binding. Mouse anti–IL-4 receptor (Genzyme, Cambridge, MA) or mouse Ig (Sigma) at a concentration of 1 μg/10⁶ cells was then added for 30 minutes. After the cells were washed three times and again blocked with rat IgG for 10 minutes, phycoerythrin (PE)-labeled goat anti-mouse Ig (Tago, Burlingame, CA) was added for 30 minutes. Cells were washed three times and analyzed using a FACScan flow cytometer (Becton Dickinson).

Measurement of DNA synthesis. The effects of TGF-β on B-cell proliferation were assessed by measuring [3H]-thymidine incorporation as previously described. Briefly, freshly isolated normal or B-CLL B cells (2 × 10⁶ in 0.2 mL) were cultured with 1:10,000 final dilution of Staphylococcus aureus Cowan strain (SAC) and 10% culture supernatant from MLA-144, with or without 5 ng/mL of TGF-β. Cultures were incubated in 96-well flat-bottom microtiter plates (Falcon, Franklin Lakes, NJ) for 3 to 5 days. Sixteen hours before termination of culture, each well was pulsed with 2 μCi [3H]-thymidine (Amersham, Arlington Heights, IL) and harvested onto fiberglass filters with a multichannel automated cell harvester (Brandel M12V; Biomedical Research Institute, Gaithersburg, MD). Filters were air dried at room temperature, transferred to plastic scintillation vials containing 2.0 mL Ecoscintfluor (ICN, Costa Mesa, CA), and counted in an automated liquid scintillation counter (Intertechnique BETAmatic; Beckman, Irvine, CA).

RESULTS

TGF-β increases apoptosis of normal B cells, but not CLL B cells. B cells were isolated from the PB of normal donors by positive selection as described. The purified B cells were placed in culture and assayed for apoptosis using the TUNEL technique. Figure 1A is a representative experiment of seven with normal B cells after 48 hours in culture. Apoptotic B

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**Table 1. Inhibition of CLL B-Cell and Normal B-Cell Proliferation by TGF-β**

<table>
<thead>
<tr>
<th>CLL B-cell donor</th>
<th>Proliferation SAC/MLA (cpm)*</th>
<th>Proliferation SAC/MLA + TGF-β (cpm)</th>
<th>Percent Inhibition†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL 1</td>
<td>4,384</td>
<td>2,910</td>
<td>34</td>
</tr>
<tr>
<td>CLL 2</td>
<td>29,180</td>
<td>21,710</td>
<td>26</td>
</tr>
<tr>
<td>CLL 3</td>
<td>9,392</td>
<td>7,581</td>
<td>19</td>
</tr>
<tr>
<td>CLL 4</td>
<td>1,612</td>
<td>610</td>
<td>62</td>
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<tr>
<td>CLL 5</td>
<td>8,118</td>
<td>1,774</td>
<td>78</td>
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<tr>
<td>CLL 6</td>
<td>21,193</td>
<td>2,322</td>
<td>89</td>
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<tr>
<td>CLL 7</td>
<td>18,662</td>
<td>8,895</td>
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<td>CLL 8</td>
<td>2,549</td>
<td>500</td>
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<td>CLL 9</td>
<td>2,294</td>
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<td>CLL 11</td>
<td>3,379</td>
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<tr>
<td>CLL 12</td>
<td>1,399</td>
<td>985</td>
<td>30</td>
</tr>
<tr>
<td>CLL 13</td>
<td>2,732</td>
<td>1,806</td>
<td>34</td>
</tr>
<tr>
<td>CLL 14</td>
<td>2,178</td>
<td>1,237</td>
<td>43</td>
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<tr>
<td>CLL 15</td>
<td>1,729</td>
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<td>CLL 16</td>
<td>18,135</td>
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<td>87</td>
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<tr>
<td>CLL 17</td>
<td>1,327</td>
<td>865</td>
<td>35</td>
</tr>
<tr>
<td>CLL 18</td>
<td>2,345</td>
<td>1,016</td>
<td>57</td>
</tr>
<tr>
<td>CLL 19</td>
<td>1,159</td>
<td>370</td>
<td>68</td>
</tr>
<tr>
<td>CLL 20</td>
<td>6,483</td>
<td>5,575</td>
<td>14</td>
</tr>
</tbody>
</table>

* Data shown were maximum proliferation: CLL B cells (3- to 5-day culture), normal B cells (3 day culture); Sixteen hours before harvest, 2 μCi [3H]-thymidine was added to each well. Proliferation was measured by [3H]-thymidine incorporation in cpm.
† Percent inhibition of proliferation was calculated as: ([Proliferation SAC/MLA – Proliferation SAC/MLA/TGF-β] × [Proliferation SAC/MLA]) × 100.
cells have increased fluorescence because of the incorporation of FITC-dUTP. In this case, 23% of the B cells in culture were apoptotic. Addition of TGF-β at initiation of the culture increased the level of apoptosis to 55%, as shown in Fig 1B. This example and the overall data described below confirm previous reports that TGF-β significantly increases the apoptosis of normal B cells.12

We then determined the effect of TGF-β on CLL B-cell apoptosis. Figure 2 compares our overall results with normal and CLL B cells. As shown in Fig 2A, spontaneous apoptosis of normal B cells was 24% ± 4% (n = 7) after 48 hours in culture. Addition of TGF-β increased the apoptosis of normal B cells to 50% ± 5% (n = 7). In contrast to normal B cells, the magnitude of spontaneous CLL B-cell apoptosis varied considerably among the patient population, ranging from 6% to 57% (Fig 2A). Except for one patient, addition of TGF-β minimally altered spontaneous apoptosis. Despite the variability in spontaneous apoptosis, Fig 2B shows clearly that CLL B cells are consistently resistant to the apoptotic effects of TGF-β (P < .002).

We then asked if this lack of response to TGF-β by the leukemic cells was dose related. Addition of up to 20 ng/mL of TGF-β did not significantly alter the level of CLL B-cell apoptosis. For example, in one case of four studied, spontaneous apoptosis of CLL B cells after 48 hours in culture was 47% and reached a maximum of only 54% when 20 ng/mL of TGF-β was added.

Interestingly, the leukemic cells were capable of increased apoptosis in response to other mediators such as hydrocortisone and anti-IgM (data not shown). Therefore, although CLL B cells are resistant to apoptosis induced by TGF-β, they do not exhibit a generalized defect to induction of apoptosis by other mediators.

To determine whether the kinetics of TGF-β mediated apoptosis of CLL B cells was altered, we quantified the number of viable cells by propidium iodide exclusion over several days. Figure 3 is representative of four cases in which addition of TGF-β had no effect on apoptosis at 48 hours. As shown, CLL B cells die rapidly when cultured with media alone. Addition of TGF-β did not alter the viable cell recovery over a seven day period (Fig 3) or alter the percentage of viable CLL B cells in culture (data not shown). Taken together, these data further demonstrate the resistance of CLL B cells to apoptosis induced by TGF-β.

It has been reported previously that TGF-β has variable effects on the usually poor proliferative response of CLL B cells to mitogenetic stimulation.14,15 We confirmed these results, finding that cells from 20 CLL patients generally proliferated weakly when stimulated with SAC/MLA (Table 1). Typically, these CLL B cells were modestly inhibited by TGF-β, although occasional cases showed marked inhibition (Table 1; mean inhibition, 49% ± 25%; range, 2% to 89%). B cells from normal donors showed increased proliferation with SAC/MLA, which was inhibited with TGF-β (Table 1; 65% ± 15%; range, 28% to 82%). Despite variable stimulation with SAC/MLA, the percent inhibition of proliferation by TGF-β on CLL and normal B cells was not significantly different (P > .05).

**Table 2. Expression of the TGF-β Type II Receptor and Production of TGF-β**

<table>
<thead>
<tr>
<th>TGF-β Type II Receptor* (fold change in MFI)</th>
<th>TGF-β Production* (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>CLL B cells</td>
<td>(mean ± SD) (n = 12)</td>
</tr>
<tr>
<td>Normal B cells</td>
<td>(mean ± SD) (n = 7)</td>
</tr>
</tbody>
</table>

* Cells or supernatants were obtained after 48 hours in culture; not significantly different, P > .05 as determined by Student’s t-test.

**Fig 4. Normal and CLL B-cell expression of the TGF-β type II receptor are similar. Normal and CLL B cells were cultured for 48 hours in media with 5% FCS. Subsequently, cells were labeled with anti-CD19-PE and anti–TGF-βRII followed by goat anti-rabbit Ig-FITC. Expression of the TGF-β type II receptor (solid histogram) is shown relative to isotype staining (open histogram) after gating on CD19** lymphocytes. Data for normal and CLL B cells are representative of 7 and 12 samples, respectively.
in 17 cases. After 48 hours in culture, production of both total and active TGF-β by normal B cells and CLL B cells was not statistically different \((P > .05)\), although the latter showed more individual variability (Table 2). The variation in production of TGF-β in individual CLL cases did not correlate with levels of apoptosis or inhibition of proliferation induced by TGF-β addition.

*IL-4 dramatically inhibits apoptosis of CLL B cells.* Because previous reports indicated that IL-4 inhibits apoptosis of CLL B cells in culture,\(^1\) we compared the effects of IL-4 on CLL B cells and normal peripheral B cells. As shown in Fig 5A, adding IL-4 moderately decreased the spontaneous apoptosis of normal B cells from 24% ± 4% to 18% ± 3% \((n = 7)\) after 48 hours in culture.

However, adding IL-4 to CLL B-cell cultures markedly diminished apoptosis in 9 of 11 patients, reducing it to less than 7% in 6 patients (Fig 5A). Figure 5B demonstrates the overall relative effect of IL-4 on spontaneous apoptosis of normal and CLL B cells. In the leukemic B cells, the ability of IL-4 to significantly reduce apoptosis was not influenced by the addition of TGF-β (data not shown), but in normal B cells, IL-4 reduced apoptosis induced by TGF-β from 49% ± 7% with TGF-β alone to 28% ± 5% with TGF-β and IL-4 \((n = 5);\) data not shown).

**Expression of IL-4 receptors by CLL and normal B cells.** To further investigate the role of IL-4 in CLL, we examined IL-4 receptor expression by the CLL B cells using flow cytometry (Fig 6). Expression of IL-4 receptors by CLL B cells was significantly increased over background fluorescence, and expression of this receptor was uniform, with a single population of positive cells (Fig 6). Table 3 compares the MFI of the IL-4 receptor on freshly isolated normal versus CLL B cells. As previously reported,\(^2\) and shown here, expression of the IL-4 receptor on freshly isolated normal B cells was minimal \((1.1 ± 0.2\text{-fold change in MFI}; n = 3)\). In contrast, CLL B cells constitutively expressed IL-4 receptors \((3.2 ± 0.8\text{-fold increase in MFI over background}; n = 4; P < .01)\). Thus, increased expression of IL-4 receptors by CLL B cells may contribute to the enhanced anti-apoptotic effect of IL-4 compared with normal B cells.

**DISCUSSION**

The pathogenesis of CLL is characterized by an accumulation of primarily noncycling CD5⁺ B cells. It has been suggested that regulation of apoptosis by cytokines could contribute to the expansion of these B cells.\(^7\) Our data support this argument and show CLL B cells respond differently to TGF-β and IL-4, compared with normal B cells. CLL B cells are resistant to the apoptotic effects of TGF-β, despite TGF-β type II receptor expression that was at a similar low level as normal B cells. In addition, nonresponsiveness of CLL B cells to TGF-β was not dependent on cytokine dose or time in culture. We also demonstrated that the Th2 cytokine IL-4 dramatically reduced spontaneous apoptosis of CLL B cells compared with normals. The anti-apoptotic effect of IL-4 may be mediated through increased expression of IL-4 receptors by CLL B cells.

Previous reports have suggested that TGF-β may be important in the development of CLL and/or in some of the related defects in these patients, although its effect on apoptosis has not been addressed in detail. TGF-β is produced by CLL B cells and present in the serum of patients.\(^13\) Production of TGF-β in CLL by stromal cells may be responsible for decreased IL-6 production and inhibition of hematopoietic precursors in patients.\(^29\) Furthermore, our data confirm previous reports that TGF-β inhibits the proliferation of CLL B cells, although the effect was variable.\(^14,15\) CLL B-cell proliferation is already blunted compared with normal B cells, and thus the relevance of further inhibition by TGF-β in CLL is unclear. Previous reports have shown that tumor
production of TGF-β can induce a shift toward Th2 cytokine production and progression of immunosuppressive states in tumor-bearing mice. 

An analogous situation is possible in CLL where preferential expression of Th2 cytokines such as IL-4 sustain leukemic cell survival, whereas TGF-β contributes to the observed clinical immunosuppression. In support of this hypothesis, we have found that the Th2 cytokine IL-5 dramatically decreases apoptosis of CLL B cells compared with normal B cells (manuscript submitted).

Escape from the growth inhibitory effects of TGF-β have been reported in other tumor systems. 

In several epithelial malignancies this appears to be mediated by inactivation of the TGF-β type II receptor. Also, in Sézary syndrome, a chronic T-cell leukemia, we demonstrated reduced surface expression of TGF-β type II receptor with resultant lack of inhibition by TGF-β, but a defect in the receptor has not been defined.

As shown in our present study, CLL B cells are resistant to the apoptotic effects of TGF-β, and this effect was probably not due to quantitative alterations in the expression of the TGF-β type II receptor, because levels were comparable to normals. Although this escape from TGF-β mediated apoptosis could be due to altered binding of TGF-β to the receptor complex or structural or quantitative alterations of the type I or III receptors, this would likely also abrogate growth inhibitory effects of TGF-β, and in our study as well as previous reports, CLL B-cell proliferation was moderately although variably inhibited by TGF-β. Taken together, these data imply that CLL B cells are capable of binding TGF-β and signaling, but downstream events that specifically regulate apoptosis may be altered.

An obvious candidate for such a downstream mediator is Bcl-2, which has been shown to reduce apoptosis in several systems. Overexpression of Bcl-2 has been reported in CLL B cells, but whether increased Bcl-2 expression is pathologic or physiologic in this circumstance remains unclear. It will be of interest to see if there are other abnormalities in gene expression in the apoptotic pathways of CLL B cells.

It is also possible that CLL B-cell resistance to TGF-β-mediated apoptosis could simply represent a response of CD5+B cells. Investigation of normal human CD5+B cells has been difficult because they are relatively rare in adults, and fetal CD5+B cells may be functionally unique. However, we have investigated this question in the autoimmune New Zealand Black (NZB) mouse, which has been proposed as a mouse model for CLL. We found that CD5 expression does not appear to determine B-cell responses to TGF-β; apoptotic responses to TGF-β were the same in purified CD5+B versus CD5+B cells isolated from the spleens of these mice (manuscript in preparation).

Previous reports have shown that IL-4 prevents apoptosis of CLL B cells and increases long-term viability, possibly by a Bcl-2 dependent pathway. However, the effect of IL-4 on apoptosis of CLL B cells compared with normal B cells has not been thoroughly examined. Our data indicate that IL-4 markedly reduced apoptosis of CLL B cells, but only moderately inhibited apoptosis of normal B cells. The enhanced anti-apoptotic effects of IL-4 on CLL B cells may be mediated through increased expression of IL-4 receptors. Previous studies have reported low expression of IL-4 receptors by resting PB B cells, whereas CLL B cells can express IL-4 receptors. In our study, we showed a significant increase in MFI of IL-4 receptor expression in CLL B cells versus normal B cells, suggesting increased numbers of receptors. To further evaluate increased IL-4 receptor expression, binding studies could be performed.

In this study, we have shown that the apoptotic responses of CLL B cells to TGF-β and IL-4 are aberrant and could provide a selective advantage for tumor expansion. Also, concurrent production of TGF-β by the expanding CLL B cells could help to explain some aspects of the extensive dysfunction of normal B cells and T cells in many of these patients.

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REFERENCES


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