Bone Marrow-Derived Stromal Cells Prevent Apoptotic Cell Death in B-Lineage Acute Lymphoblastic Leukemia

By Atsushi Manabe, Elaine Coustan-Smith, Frederick G. Behm, Susana C. Raimondi, and Dario Campana

Establishing requirements for the survival of human immature B cells in vitro has proved elusive. In this article, we report prolonged survival of B-lineage leukemic cells on "feeder layers" of bone marrow (BM)-derived stromal cells in a serum-free environment. In 15 of 18 cases of B-lineage acute lymphoblastic leukemia (ALL), there was a greater than 50% decrease in the number of viable cells after 72 hours of culture in medium alone. Cell loss was preceded by molecular and cellular changes characteristic of programmed cell death, or apoptosis, and was not suppressed by adding interleukin-7 to the tissue culture medium. By contrast, the use of allogeneic BM stromal cells as feeder layers prevented apoptosis in 10 of 12 cases of ALL, leading to extended survival of the blast cells. This method was not successful when the allogeneic marrow cells were replaced with established cell lines. In six of eight cases in which the numbers of intact CD19⁺ lymphoblasts were counted by flow cytometry after 7 days of culture, the proportion of such cells recovered in the presence of BM stromal cells was 68.8% to 124.7% (median, 95.3%) of that originally seeded, as opposed to the 0.3% to 15.9% fraction (median, 0.7%) obtained in the absence of stromal cells. Survival requirements of the B-lineage lymphoblasts appeared to be heterogeneous, as cells from 3 of the 18 cases studied showed no signs of apoptosis in serum-free tissue culture medium that lacked BM stromal cells. The only cells not giving rise to viable cultures were from two hyperdiploid (>50 chromosomes) cases with identical karyotypes. The serum-free assay described here can be used to compare the survival requirements of normal and leukemic B-cell progenitors as well as to identify the molecules involved in the interaction between BM stroma and immature B cells.

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In some cases, the addition to the tissue culture medium of purified factors, such as low molecular weight B-cell growth factor (LMW-BCGF) or recombinant cytokines, such as IL-7 and/or IL-3, has been reported to increase colony formation, thymidine incorporation, or both. In those experiments, however, cell counts often decreased precipitously, despite apparently vigorous DNA synthesis. Thus, although these methods have led to the identification of some factors that may interact with leukemic blasts, the minimal requirements for survival of leukemic lymphoblasts in vitro remain to be defined.

Long-term survival and expansion of normal murine and human hematopoietic progenitors appears to depend on the presence of bone marrow (BM) stromal cells. Murine B-cell progenitors require BM stromal cells to expand in the culture system developed by Whitlock and Witte; no equally successful technique has been standardized for human B cells, although BM stromal cells may be necessary to support the generation of CD19⁺ BM cells and the formation of human BM B-cell colonies. Whether or not stromal cells can support the in vitro survival of blast cells from cases of B-lineage ALL has not been thoroughly investigated. Of particular interest are the survival requirements of ALL subtypes: are they similar or do they differ according to properties of the blast cells?

We have observed that when B-lineage ALL cells are placed in culture, signs of programmed cell death, or apoptosis, become rapidly apparent in most cases. Consequently, we tested several approaches to blocking this process and found that it could be prevented by seeding leukemic blasts onto BM-derived stromal cells in a serum-free medium. We report here results showing the reproducibility of this method for maintaining B-lineage ALL cells in vitro.

MATERIALS AND METHODS

Cells. BM and peripheral blood (PB) samples were collected at diagnosis from 18 patients, aged 1 to 13 years (median, 6), with B-lineage ALL. In all cases, greater than 90% of the blasts were CD19⁺, CD22⁺, class II⁺ and positive for terminal deoxynucleotid-
were CD19+, and in 8 of 18 they expressed cytoplasmic μ heavy chains (Table 1). Mononuclear cells were collected after centrifugation on a density gradient (Lymphoprep; Nycomed, Oslo, Norway) and washed three times in phosphate-buffered saline (PBS) and once in AIM-V medium (GIBCO, Grand Island, NY; Cat. No. 320-2055AJ). Fresh leukemic cells were placed in culture within 4 hours from collection. In some experiments, cryopreserved samples were used immediately after thawing (Table 1). The cells viability consistently exceeded 95% by trypan-blue dye exclusion.

To obtain BM stromal cells,15 we collected mononuclear cells from two normal adult BM donors (aged 35 and 44) and three children with malignancies (one astrocytoma, two acute myeloid leukemias) but no apparent BM involvement. The cells were separated as described above, washed three times in RPMI-1640 (Whittaker Bioproducts Inc, Walkersville, MD; Cat. No. 14-901A) and resuspended at 2 × 10^6/mL in Fischer’s medium (GIBCO; Cat. No. 320-1475AJ) containing 5% fetal calf serum (FCS; Whittaker Bioproducts Inc; Cat. No. 14-901A; Lot Nos. 9M0326 and 9M0957), 15% horse serum (GIBCO; Cat. No. 200-6050PJ; Lot No. 31p7704), transferrin (Sigma, St Louis, MO; Cat. No. T-1147; 0.4 mg/mL), hydrocortisone (Sigma; Cat. No. H-0888; 10⁻⁶ mol/L), L-glutamine (Whittaker Bioproducts Inc; Cat. No. 176054 2 mmol/L), 2-mercaptoethanol (Sigma; Cat. No. M6250; 0.4 mg/mL), and penicillin-streptomycin (Whittaker Bioproducts Inc; Cat. No. 17602A). Ten-milliliter aliquots of the suspension were distributed in 25-cm² flasks (Nunc, Roskilde, Denmark; Cat. No. 163771) and placed in an incubator set at 33°C and 90% humidity. Cells were fed every 3 to 7 days by replacing 50% to 75% of the supernatant with fresh Fischer’s medium with additives. After 4 to 6 weeks of culture, a confluent layer of stromal cells appeared. Cells were then detached by treating with trypsin (Whittaker Bioproducts Inc; Cat. No. 17-161A) and resuspended in fresh Fischer’s medium containing additives, and distributed into a 24-well plate (Costar, Cambridge, MA; Cat. No. 3424). Confluent stromal cells were seen in the wells after 1 to 2 weeks of culture.

The human fibroblastic cell line W18Va20 was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Whittaker Bioproducts Inc; Cat. No. 12-614B) containing 10% FCS, L-glutamine, penicillin, and streptomycin. The M2-10B4 murine fibroblast-like cell line of BM stromal origin,21 a gift of Dr C.J. Eaves (Terry Fox Laboratory, B.C. Cancer Research Center, Vancouver, Canada), was cultured in RPMI-1640, whereas the human BM stromal line KM-102,22 a gift of Dr K. Harigaya (Chiba University, Japan), was kept in Jacove’s modified Dulbecco’s medium (IMDM; GIBCO; Cat. No. 380-1440AJ; both tissue culture media were supplemented with FCS, L-glutamine, and antibiotics. Cell lines were kept in an incubator set at 37°C and 5% CO₂ with 90% humidity. For the experiments described below, cells were detached with trypsin and distributed in 24-well tissue culture plates as above. In some experiments, the cells in the plates were irradiated with a 1³⁷Cs cesium source at 2,000 cGy (750 cGy/min). After irradiation, the cells were kept in an incubator as above until the experiments were performed.

### Table 1. Characteristics of Cases of B-Lineage ALL

<table>
<thead>
<tr>
<th>Case No.*</th>
<th>Patient’s Age (yr)</th>
<th>Immunophenotype</th>
<th>Karyotype</th>
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<tr>
<td>1</td>
<td>10</td>
<td>CD34⁻, CD10⁻, cμ⁺</td>
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<td>2</td>
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<td>11</td>
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</tr>
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<td>4</td>
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<tr>
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<td>CD34⁺, CD10⁻</td>
<td>46,XX</td>
</tr>
<tr>
<td>7</td>
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</tr>
<tr>
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<td>1</td>
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</tr>
<tr>
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<td>5</td>
<td>CD34⁺, CD10⁻, cμ⁺</td>
<td>46,XY,del(11)(q23)</td>
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</tbody>
</table>

*Samples 14 to 17 were cryopreserved at diagnosis and later thawed for use in this study. The remaining samples were used immediately after collection.

In all cases, greater than 90% of blasts were positive for CD19, CD22, class II, and TdT antigens; the requirement for CD34, CD10, and cμ positivity was greater than 90% reactive cells.
by vigorously pipetting and scraping the bottoms of wells with a plastic pipette. The lack of residual cells in the wells was always confirmed by observation with an inverted microscope. After one wash in PBS, the cells were passed through a 19-gauge needle to disrupt clumps observed in the samples containing fibroblasts or BM stromal cells and washed once again in PBS.

**DNA gel electrophoresis.** In this study we used the assay described by Sellins and Cohen,25 with minor modifications. Mononuclear cells (0.5 to 2 × 10⁶) were centrifuged at 500g for 10 minutes. The cell pellet was lysed by adding 0.5 mL of hypotonic lysing buffer (10 mmol/L Tris, pH 7.4; 1 mmol/L EDTA; 0.2% Triton X-100) to CD19+ cells not treated with steroids. We observed that DNA fragments of multiples of 180 bp that passed through the nozzle in 30 seconds was recorded. Thus, only morphologically intact, CD19+ lymphoid cells were counted. This method yielded reliable estimates of different proportions of target cells present in artificial mixtures of different cell types.

For immunophenotypic studies, cells were labeled as above with the following additional MoAbs: W6/32 (CD10; BD; Cat. No. 7500); LeuM7 (CD13; BD; Cat. No. 7830) and LeuM9 (CD33; BD; Cat. No. 7780) followed after two washes in PBSA by goat antiserum to mouse IgG conjugated to FITC (Jackson, West Grove, PA; Cat. No. 115-095-003); Leu4 (CD3) conjugated to phycoerythrin (PE; BD; Cat. No. 7349). Isotype-matched nonreactive MoAbs (BD) were used as a control.

**RESULTS**

**Leukemic cells from most cases of B-lineage ALL die of apoptosis in vitro.** When samples of B-lineage ALL were placed in serum-free tissue culture medium, more than 50% of the viable (trypan-blue negative) cells were lost by 72 hours of culture in 15 of the 18 cases studied. This loss was paralleled by a drastic reduction of CD19+ cells with the light-scattering properties of the corresponding diagnostic sample, as determined by flow cytometry. In the remaining three cases (nos. 9, 14, and 18, Table 1), greater than 99%, 55%, and 95% of cells were viable after 72 hours, respectively.

The disappearance of viable leukemic cells was preceded by molecular and cellular changes characteristic of apoptosis.19 First, the DNA fragmentation assay, performed after 24 to 72 hours of culture, showed the typical ‘ladder’ of multiples of 180-bp fragments in cases with losses of viable cells (Fig 1). The intensity of the ladder was similar or higher to that observed when analyzing preparations containing 30% to 50% of steroid-treated CEM-C7 cells admixed with cells cultured without dexamethasone (see Materials and Methods). By contrast, no DNA fragmentation was seen in one sample in which the numbers of viable cells did not decrease with time in serum-free medium. Second, progressive changes in the cells’ morphologic features—such as a decreased nuclear/cytoplasmic ratio, chromatin condensation, and nuclear fragmentation—were seen after Wright-Giemsa staining (Fig 2). Third, changes in the cells’ light-scattering properties (eg, a lower forward light scatter and a higher side scatter, indicating a smaller size and a higher granularity) were also consistent with apoptosis (Fig 3).

**Human BM-derived stromal cells prevent apoptosis of B-lineage leukemic cells.** In most instances, apoptosis appeared to be completely abolished by placing the leukemic cells on allogeneic BM stromal cells. In 10 cases (nos. 1, 2, 4 through 8, 10, 11, and 15, Table 1) of the 12 studied, there was no evidence of DNA fragmentation (Fig 1) or morphologic signs of apoptosis after 24 to 72 hours of culture, in contrast to findings in the corresponding control samples. In two samples (nos. 12 and 13, Table 1), apoptosis was evident despite the presence of BM stromal cells.

Because these observations could have been caused by the elimination of apoptotic cells by macrophages, we next investigated the effects of BM stromal cells on the numbers of leukemic cells after different periods of time. Using flow cytometry, we found that, in the presence of BM stromal...
cells in the BM stromal cultures remained stable for 12, 7, and 6 weeks, respectively, after which time the cultures were deliberately terminated. Cells in culture appeared to be intimately associated with, and in some areas surrounded by, fibroblast-like cells. When leukemic cells were maintained in culture for more than 2 weeks, the stromal cells began to detach and form clumps. In those cases, BM stromal and leukemic cells were collected by vigorous pipetting, passed through a 19-gauge needle, and reseeded onto freshly confluent BM stromal cells. This procedure was repeated every 2 to 3 weeks. In selected cases, additional phenotypic tests were performed. The phenotypic features of case 1 after 8 weeks of culture are illustrated in Fig 4. The leukemic blast cells retained CD19 and CD10 positivity and remained negative for CD13, CD33, and CD3.

**Effects of IL-7, FCS, and stromal cell lines on the survival of leukemic B-cell progenitors.** Cell loss due to apoptosis was not prevented by adding IL-7 to the tissue culture medium at a final concentration of 25 ng/mL (Figs 1, 3, and 5). Similarly, addition of either of two different lots of FCS at a final concentration of 10% had no apparent effect on apoptosis in two of three cases studied (nos. 1 and 6, Table 1; see also Fig 5). These preparations were selected from 12 different lots and had the lowest (FCS ‘A’) and the highest (FCS ‘B’) spontaneous mitogenic activities when tested with PB lymphocytes (data not shown). FCS ‘A’ is currently used in our laboratory for colony assays with normal myeloid and erythroid cells. In a third case (no. 7, Table 1), the addition of FCS to the tissue culture medium had a noticeable effect on the blast cells’ survival. The proportions of cells recovered after 7 days of culture with FCS ‘A’ and FCS ‘B’ in this case were 22.9% and 97.3%, respectively (Fig 5).

Finally, we tested the ability of cell lines to substitute for human allogeneic BM stromal cells. Irradiated or nonirradiated murine BM stromal M2-10B4 cells were used in four cases (nos. 1, 6, 8, and 10), human fibroblastic W18Va2 in two (nos. 1 and 8), and human BM stromal KM-102 in two (nos. 6 and 8). In all experiments, the cell lines failed to sustain the viability of leukemic cells, and after 7 days of culture the number of leukemic cells recovered was invariably less than 20% of that originally seeded. These findings confirm the specificity of the activity of nontransformed BM stromal cells. Moreover, cell lines used as a ‘feeder layer’ appeared to be more susceptible to detachment from the bottom of the wells after leukemic cells were seeded than did the allogeneic BM stromal cells. In two of the four experiments performed with the M2-10B4 line, and in one of the two performed with the W18Va2 line, feeder layers had partially detached after 7 days of culture.

**DISCUSSION**

Hematologists have long been frustrated by the difficulty of culturing blast cells freshly taken from cases of B-lineage ALL. We first addressed this issue by attempting to maintain leukemic cells in culture using serum-free tissue culture medium that had proved suitable for sustaining the growth of cell lines and lymphoid proliferation after mito-
Morphologic signs of apoptosis are prevented by BM stromal cells. Cells from a case of B-lineage ALL (no. 8, Table 1) were kept in vitro for 48 hours in the absence (A) and in the presence (B) of BM stromal cells. Cytocentrifuge preparations were stained with Wright-Giemsa. Different stages of morphologic degeneration consistent with apoptosis, such as cell shrinkage, chromatin condensation, and nuclear fragmentation, are visible in (A). Morphologic changes were abolished by seeding cells onto allogeneic BM stromal cells (B).

In our experiments, DNA fragments could be seen as early as after 24 hours of culture, when the surface membrane of most cells was still impermeable to trypan blue or propidium iodide. Conceivably, this process may begin even earlier, as reported by Baxter et al for two cases of B-lineage ALL.

In the present study, we showed that signs of apoptosis could be abolished by seeding B-lineage leukemic cells onto...
Table 2. Quantitation of CD19+ Leukemic Lymphoid Cells in Culture

<table>
<thead>
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<th>Sample</th>
<th>Day 0</th>
<th>Controlt</th>
<th>Stroma*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>6</td>
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<td>63 (0.7)</td>
<td>8,435 (94.1)</td>
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<td>7</td>
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<td>18 (0.3)</td>
<td>6,865 (96.5)</td>
</tr>
<tr>
<td>8</td>
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<td>28 (0.3)</td>
<td>5,973 (68.8)</td>
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<tr>
<td>10</td>
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</tr>
<tr>
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<td>355 (3.8)</td>
<td>7,185 (76.9)</td>
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<tr>
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<td>9,622</td>
<td>47 (0.5)</td>
<td>149 (1.5)</td>
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<tr>
<td>13</td>
<td>8,571</td>
<td>130 (1.5)</td>
<td>2,608 (30.4)</td>
</tr>
</tbody>
</table>

*See Table 1.
†Cells kept in culture with serum-free medium only.
‡Cells seeded onto allogeneic BM stromal cells. For cases no. 1, 6, and 8, the cell numbers remained unchanged for 12, 7, and 6 weeks, respectively.
§Numbers of CD19+ cells with lymphoid morphology passing through the nozzle of the flow cytometer in 30 seconds (see text).
||Percentage of cells recovered after 7 days.

Results are expressed as the means of two experiments performed with two different samples of BM stromal cells.

BM stromal cells. By measuring cell numbers at different times during culture with multiparameter flow cytometry, and comparing the cells' phenotype and morphologic features with those recorded at the beginning of the culture, we were able to establish that loss of leukemic cells had indeed been prevented. The advantages of this approach are clear. First, the cells counted are phenotypically characterized and no irrelevant cells (eg, T lymphocytes) are included. Second, even minor morphologic changes that accompany apoptosis can be detected, and cells with such features can be excluded from the count.

In contrast to findings by Umiel et al. that only autologous BM stromal cells would support B-lineage leukemic progenitors, our allogeneic system supported leukemic cells for over 12 weeks. However, we cannot exclude the possibility that extended survival (eg, > 10 months) may require autologous feeder layers, as reported by Umiel et al.

The observation that IL-7 did not improve the survival of leukemic blasts may be confusing in view of recent reports indicating that this cytokine is not only a primary growth-promoting factor for normal murine and human B-cell progenitors, but it can also stimulate thymidine incorporation in some cases of B-lineage ALL. For instance, increased DNA synthesis was reported by Eder et al. in 3 of 10 B-lineage ALL cases cultured for 7 days in the presence of IL-7, and by Masuda et al. in two of seven cases cultured with IL-7 for 3 days. However, in the first study, Eder et al. reported a drastic reduction in cell numbers (80% in some experiments) despite the presence of IL-7. These results suggest different mechanisms of leukemic cell survival and proliferation. It remains to be tested whether the addition of IL-7 would stimulate the expansion of the leukemic cell population in the presence of BM stroma, as observed with murine pro-B-cell clones.

BM stromal cells synthesize several cytokines, including transforming growth factor-β (TGF-β), colony-stimulating...
factor-1 (CSF-1), IL-6, IL-7, and “stem-cell factor” (SCF), and appear to provide optimal support for the growth and differentiation of immature B cells. In the present study, we did not address whether the beneficial action of BM stromal cells on B-lineage leukemic cells was mediated through soluble factors or cell-cell contact. In preliminary experiments, we found that the survival of leukemic blasts on polycarbonate filters was better overall if the filters were placed in wells containing BM stromal cells. In some cases, close proximity between the ALL cells and stroma was necessary for long-term maintenance in culture.

Thus, B-lineage leukemic progenitors appear to have heterogeneous survival requirements, as also demonstrated by the variable response to different batches of FCS.

BM stroma is a complex structure composed of different cell types. In our study, established human fibroblastic and BM stromal cell lines were not suitable to support B-lineage ALL cells, although the latter cells and their conditioned medium can support granulo-monocytic and erythroid colonies. Preliminary results of studies to test the activity of BM stromal cells prepared in the absence of hydrocortisone and containing no adipocytes showed that this system would support B-lineage leukemic cells, but not as well as the “classical” BM stromal cells (A. Manabe, D. Campana, unpublished observations). Earlier reports indicating the possibility of using murine cells to support the growth of human cell lines, as well as the documented feasibility of engrafting immune-deficient mice with cells from selected cases of human leukemia, prompted us to test the effects of the M2-10B4 murine BM stromal line. However, despite the ability of this line to support the growth of murine pre-B cells, it failed to sustain the survival of human leukemic cells in our system. Hence, we are still uncertain as to the stromal cell types that contribute most to the survival of leukemic B-cell progenitors.

In general, it was not possible to correlate the phenotypic features of the leukemic cells with their ability to survive in culture. Among the cases in which BM stromal cells provided critical survival support, the blast cells represented a spectrum of maturational levels and cytogenetic changes. Notable exceptions were two cases of hyperdiploid ALL (>50) with identical karyotypes that could not be maintained with stromal cells. This finding is consistent with the exquisite chemosensitivity of such leukemias and parallels the observation of a lower ‘plating efficiency’ for hyperdiploid B-lineage ALL in colony assays. However, there was one other hyperdiploid case in which the blast cells survived well on stromal feeder layers (no. 10, Tables 1 and 2). We speculate that the assessment of leukemic blast cell survival in vitro, using the system described in this report, may show how individual cases resist adverse conditions and thus could provide important prognostic information.

The reproducibility of our system is facilitated by the commercial availability of serum-free and cytokine-free tissue culture medium sufficient to sustain mammalian cell growth. The success of the method appears independent of the source of BM stromal cells, because no differences in capacity to support cell survival were apparent among the five different allogeneic stromal preparations tested. This approach can now be used to identify similarities and discrepancies between the survival requirements of normal and malignant B cells. In addition, the molecules involved in the interaction between BM stromal and leukemic cells can be studied, by assessing, for instance, cell survival after the addition of antibodies against cell surface antigens and cytokines. Definition of the factors that determine leukemic cell survival would have both theoretical and practical implications. For example, the reliability of currently used drug-sensitivity assays is limited by the massive leukemic cell death that occurs even in the absence of drugs. The techniques established in this study appear eminently suitable for such assays.

ACKNOWLEDGMENT

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REFERENCES


Bone marrow-derived stromal cells prevent apoptotic cell death in B-lineage acute lymphoblastic leukemia

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