Anti-CD3 + Interleukin-2 Stimulation of Marrow and Blood: Comparison of Proliferation and Cytotoxicity

By Peter M. Anderson, Augusto C. Ochoa, Norma K.C. Ramsay, Diane Hasz, and Daniel Weisdorf

The proliferation and in vitro cytolytic activity of interleukin-2 (IL-2)-activated and anti-CD3+IL-2-stimulated marrow mononuclear cells (MMC) and peripheral blood mononuclear cells (PBMC) were studied. Samples from 8 normal donors, 15 patients with acute lymphoblastic leukemia (ALL), and 7 patients with non-Hodgkin’s lymphoma (NHL) in remission were cultured in IL-2 (100 U/mL) or IL-2 (100 U/mL) plus anti-CD3 (10 ng/mL). MMC as well as PBMC samples demonstrated significant synergy between IL-2 and anti-CD3 in the promotion of proliferation as measured by 3H thymidine incorporation on day 5 (P < .001) or fold increase in cell number on day 14. Cryopreserved marrow specimens had equally rapid proliferation as fresh MMC when cultured in the presence of anti-CD3+IL-2. Anti-CD3 concentrations of 3, 11, 33, and 100 ng/mL augmented proliferation similarly in the presence of IL-2 (0.1 to 100 U/mL). Mean fold increase in cell number of both marrow- and blood-derived cultures after 14 days were significantly higher for anti-CD3+IL-2–stimulated cultures compared with cultures stimulated with IL-2 only (50- to 200-fold increase in cell number; P = .01). Comparison of remission MMC and PBMC from ALL and NHL patients with normal controls showed equivalent growth rates of activated cultures at 7, 14, and 21 days. Marrow purging with immunotoxin anti-CD19 pokeweed antiviral protein plus 4HC had no significant effect on proliferation of anti-CD3+IL-2–stimulated MMC cultures in patients with ALL.

Cytolytic activity of IL-2– and IL-2+anti-CD3–activated PBMC and MMC cultures was assessed in 51Cr release assays using K562 (natural killer [NK]-sensitive), Daudi (Burkitt’s lymphoma-, NK-resistant), and Nalm-6 (ALL-, lymphokine-activated killer [LAK]-resistant) cell lines and cryopreserved ALL blasts. Cytolytic activity on a per-cell basis [percent cytotoxicity at an effector:target ratio of 30:1] was similar in IL-2–activated PBMC- and MMC-derived cultures from ALL patients. MMC activated with anti-CD3 plus IL-2 killed Daudi significantly less well than IL-2–activated cultures on days 12 and 19 (P = .03); no significant differences were observed in lysis of LAK-resistant Nalm-6 or cryopreserved ALL blast targets. Dose response of anti-CD3 augmentation of Daudi and Nalm-6 killing was different in IL-2– and IL-2+anti-CD3–stimulated cultures. In the presence of phytohemagglutinin (PHA), both IL-2– and anti-CD3+IL-2–activated cultures developed lectin-dependent cellular cytotoxicity (LDCC) against LAK-resistant targets. Thus, anti-CD3+IL-2 activation of either PBMC or MMC contributes not only to significantly increased proliferation, but also generates populations of T cells with high cytolytic potential. Cryopreserved marrow may be an acceptable alternative source of anti-CD3+IL-2–stimulated cells for future immunotherapy protocols in conjunction with marrow transplantation.

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marrow from patients with ALL or NHL using anti-CD3+IL-2 stimulation.

MATERIALS AND METHODS

Patient samples. After informed consent, patients with ALL in remission (n = 15) or NHL (n = 7) and normal volunteer donors (n = 8) had marrow aspirated from the posterior iliac crest and blood collected into heparinized syringes. Mononuclear cells were isolated by dilution 1:1 in Hank's Balanced Salt Solution (HBSS) and layering the specimen over Ficoll-Hypaque (Pharmacia, Piscataway, NJ), centrifugation at 1,500g X 25 minutes, then washing of the cells obtained from the interface with RPMI 1640 with 25 mmol/L HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine (GIBCO, Grand Island, NY) and 5% heat-inactivated pooled human serum.

Cryopreservation and thawing of mononuclear cells. Samples were placed in media at a concentration of 10^7 to 5 x 10^8 cells/mL with 20% human serum and 10% dimethyl sulfoxide (DMSO) and frozen at a controlled rate to -70°C before storage in liquid nitrogen. Cells were thawed by rapid immersion and gentle agitation of a vial of cells in a 37°C water bath, then washing with media containing 10 μg/mL DNAase (Sigma, St Louis, MO; no. D5025) to prevent clumping, then washing in media without DNAase x 2.

Anti-CD3 + IL-2 stimulation for synergy studies. Mononuclear cells were diluted to 5 x 10^5 cells/mL in media containing anti-CD3 (OKT3, Ortho, Raritan NJ; 0 to 100 ng/mL for synergy experiments; otherwise 10 ng/mL) and recombinant human IL-2 (Hoffmann La Roche, Nutley, NJ; 0 to 1,000 U/mL in synergy experiments; otherwise 100 U/mL). Cells were counted and diluted every 3 to 4 days to 5 x 10^5 cells/mL to maintain densities <2 x 10^6 cells/mL. Short-term anti-CD3 stimulation was performed by addition of anti-CD3 to the mononuclear cells, then washing in media x 2 at the specified time (1 to 18 hours). Cells were then cultured in media containing IL-2.

Estimation of proliferation. Proliferation was estimated by plating 5 x 10^3 cells in a volume of 100 μL in triplicate in a 96-well microtiter plate, pulsing with 25 μL of 1:50 dilution of [3H] thymidine (1 mCi) (Amersham, Arlington Heights, IL; 0.4 μCi/well) on specified days. Wells were harvested onto filter paper discs after 6 hours for scintillation counting. Direct cell counts were done to determine fold expansion of each culture.

Cytotoxicity assays. 51Cr release microcytotoxicity assays were performed in 96-well microtiter plates using 300 51Cr-labeled tumor targets per well and effector: target ratios of 30:1, 10:1, 3.3:1 and 1:1 by serial threefold dilution of the first row before the addition of radiolabeled targets. Plates were centrifuged 5 minutes at 500 rpm to ensure cell contact, incubated 4 hours at 37°C, then centrifuged at 1,000 rpm. One hundred microliters of supernatant was harvested into glass scintillation vials before liquid scintillation counting. Malignant target included cell lines W62 (chronic myelogenous leukemia [CML], natural killer [NK]-sensitive), Daudi (NK-resistant), and Nalm-6 (ALL; LAK-resistant) and cryopreserved LAK-resistant blasts from a B-lineage ALL patient in relapse (GS ALL blasts). In some experiments, 51Cr release cytotoxicity assays were also performed using anti-CD3 (0 to 1,500 ng per well) to activate cytolytic function or PHA-P (Sigma L-9132) at a final concentration of 30 μg/mL to assess lectin-dependent cellular cytotoxicity (LDCC).

Statistical analysis. Fold proliferation and cytotoxicity of individual samples obtained from peripheral blood or marrow and then stimulated with IL-2 or IL-2 plus anti-CD3 were analyzed using descriptive statistics and paired and unpaired t-tests as calculated using Statworks version 1.1 software (Cricket Software, Malvern, PA).

RESULTS

Proliferative capacity of fresh versus cryopreserved marrow. To assess the proliferative capacity of fresh and cryopreserved marrow, we measured the DNA synthesis and growth rates after short-term stimulation with anti-CD3+IL-2. In some experiments, proliferation of cryopreserved cells was improved compared with fresh cells. Figure 1 shows a representative experiment in which the 3H thymidine incorporation into DNA as measured following initial overnight anti-CD3 stimulation and 5 days of culture in IL-2. At all concentrations of IL-2 tested cryopreserved marrow had equal or superior 3H thymidine incorporation into DNA.

Time required for stimulation of marrow proliferation by anti-CD3. The time required for effective stimulation of cryopreserved marrow with anti-CD3 is brief. As shown in Fig 2 no difference was observed in cultures stimulated 1, 3, and 18 hours with 10 ng/mL anti-CD3 before washing and plating in media containing IL-2 (10 U/mL). However, stimulation without anti-CD3 (ie, with IL-2 only) resulted in only 1/3 the levels of proliferation of anti-CD3-stimulated cultures.

Synergy between IL-2 and anti-CD3 in augmentation of marrow proliferation. The presence of both anti-CD3 and IL-2 in cultures of cryopreserved marrow mononuclear cells (MMC) facilitated proliferation in a synergistic and dose-dependent manner (Fig 3). Significant proliferation occurred with IL-2 alone only at concentrations in excess of 3 U/mL. Although all concentrations of anti-CD3 tested markedly augmented proliferation, the most prominent effects of low-dose anti-CD3 activation occurred at concen-
Anti-CD3 stimulation of cryopreserved MMC before washing: effect of time. Anti-CD3 (10 ng/mL) markedly augmented proliferation of cultures of MMC in 10 U IL-2/mL whether cells were stimulated 1, 3, or 18 hours before washing in media and culturing in IL-2. Data depict $\Delta^{3}$H thymidine incorporation in three replicate wells after 5 days of culture; error bars indicate the maximum standard deviation of the triplicate samples.

Effect of cell density on activation of MMC with anti-CD3. Figure 4 depicts the effect of cell density on proliferation of anti-CD3 activated MMC. After 18 hours incubation of MMC with 10 ng/mL anti-CD3 before washing and plating cells into culture, significantly superior proliferation was observed comparing peripheral blood versus marrow-derived cultures of 22 patient samples (paired t-test MMC versus PBMC cultured in IL-2 only $P = .76$, .11, .24 on days 7, 14, 21, respectively; paired t-test MMC versus PBMC cultured in anti-CD3+IL-2 $P = .78$, .40, and .09 on days 7, 14, and 21, respectively). Also normal control samples, ALL, and NHL patient samples had similar patterns of proliferation. Most rapid proliferation was observed between days 7 and 14 for anti-CD3+IL-2-stimulated cultures. The use of anti-CD3 in addition to IL-2 facilitated 50- to 200-fold higher increases in cell number than IL-2 alone.

Mean fold increases in cell number of B-lineage malignancy patient samples (n = 22) were significantly higher for cultures stimulated with anti-CD3+IL-2 compared with IL-2 only (paired t-test of IL-2 v anti-CD3 plus IL-2 patient samples: $P = .006$, .001, and .01 for PBMC on days 7, 14, and 21, respectively; $P = .02$, .05, and .006 for MMC on days 7, 14, 14, and 21, respectively). As shown in Table 2, comparison of marrow versus peripheral blood proliferation.

Table 1. IL-2 and Anti-CD3 + IL-2 Augmented Proliferation of PBMC and MMC

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Median Fold Increase in Cell Number (SEM)</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls (N = 8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMC IL-2</td>
<td>1.1 (0.2)</td>
<td>2.4 (1.9)</td>
<td>3.2 (1.3)</td>
<td></td>
</tr>
<tr>
<td>MMC IL-2</td>
<td>0.7 (0.1)</td>
<td>0.7 (0.1)</td>
<td>0.6 (0.1)</td>
<td></td>
</tr>
<tr>
<td>PBMC IL-2 + anti-CD3</td>
<td>10.3 (15)</td>
<td>83 (128)</td>
<td>355 (71)</td>
<td></td>
</tr>
<tr>
<td>MMC IL-2 + anti-CD3</td>
<td>12.7 (5.8)</td>
<td>217 (92)</td>
<td>214 (1,065)</td>
<td></td>
</tr>
<tr>
<td>B lineage malignancy (ALL + NHL; N = 22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMC IL-2</td>
<td>0.6 (0.15)</td>
<td>1.4 (0.4)</td>
<td>3.1 (1.5)</td>
<td></td>
</tr>
<tr>
<td>MMC IL-2</td>
<td>0.8 (0.1)</td>
<td>1.0 (0.1)</td>
<td>1.2 (0.4)</td>
<td></td>
</tr>
<tr>
<td>PBMC IL-2 + anti-CD3</td>
<td>4.6 (1.9)</td>
<td>146 (54)</td>
<td>470 (364)</td>
<td></td>
</tr>
<tr>
<td>MMC IL-2 + anti-CD3</td>
<td>2.7 (2.2)</td>
<td>70 (61)</td>
<td>230 (100)</td>
<td></td>
</tr>
</tbody>
</table>
no significant difference was observed for anti-CD3+ IL-2-stimulated MMC cultures from ALL patient marrows obtained before versus after antileukemia (anti-to remove B cells and leukemia cells (n = 8; paired t-test \( P = .424, .201, \) and .708 on days 7.14, and 21, respectively).

**Comparison of marrow versus blood lysis of tumor targets.**

Cytotoxicity against three different cell hematologic tumor lines by MMC and PBMC IL-2- or IL-2+anti-CD3-activated cultures were examined 12 and 19 days (Table 3). No significant differences between MMC and PBMC as sources of IL-2-activated cells were observed. Cytotoxicity of PBMC- and MMC-derived IL-2+anti-CD3-stimulated day 12 blood- and marrow-derived effectors against K562 (47.2%, SE 4.0 v 40.1%, SE 5.3) were not significantly different (paired t-test \( P = .199 \)). However, the NK-sensitive K562 target was significantly more easily killed on day 19 by IL-2+anti-CD3-stimulated PBMC-derived cultures (48.8%, SE 5.0 v 35.9%; SE 5.7) compared with marrow-derived cultures (paired t-test \( P = .01 \)).

The lysis of Daudi by day 12 IL-2+anti-CD3-stimulated PBMC (39.8%, SE 8.4) was significantly greater than marrow-derived effectors (30.7%, SE 6.7, paired t-test \( P = .04 \)). On day 19 after IL-2+anti-CD3-activation the mean percent cytotoxicity of Daudi was 32.5% versus 21.1% for PBMC- versus marrow-derived effectors (paired t-test \( P = .03 \)). Also, IL-2+anti-CD3-activated marrow cultures killed Daudi significantly less well than IL-2-activated cultures (\( P = .03 \) and \( P = .02 \) on days 12 and 19, respectively). As shown in Table 3, the lysis of the ALL target, Nalm-6, by these effectors was in general low. Nalm-6 lysis by day 12 IL-2+anti-CD3-stimulated PBMC (4.6, SE 1.9) was not significantly different than marrow-derived effectors (12.9%, SE 4.6, paired t-test \( P = .207 \)). Similarly, on day 19 no significant differences in Nalm-6 lysis were observed between IL-2+anti-CD3-stimulated effectors (12.9%, SE 3.6 v 15.3% SE 5.9; paired t-test \( P = .582 \)). Nevertheless, because the number of cells resulting from anti-CD3+IL-2 activation is 50- to 200-fold more than IL-2 activation, the total cytolytic potential of this scheme is greater than using IL-2 alone.

**Functional differences between IL-2 only versus anti-CD3 plus IL-2 stimulation.**

Differences in cytolytic function of IL-2-activated cultures and anti-CD3+IL-2-activated cultures were also studied using two methods to augment lytic activity against hematologic tumor targets. When anti-CD3 was added to the cytolytic assay, increases in the cytotoxicity of the cultures were not only dose- and target-dependent, but also related to the method of activation (Fig 5). One and 5 ng anti-CD3/well significantly stimulated day 5 anti-CD3+IL-2-activated effectors to kill Nalm-6 targets (Fig 5, top; no anti-CD3 v 1 and 5 ng/mL t-test \( P = .004 \) and .002, respectively). Daudi targets were increasingly susceptible to lysis by anti-CD3+IL-2-activated effectors only at much higher concentrations of anti-CD3 (Fig 5, bottom; anti-CD3+IL-2 v IL-2 activation at 500 and 1,500 ng/mL t-test \( P = .009 \) and .001, respectively). IL-2-activated effectors had no augmentation of either Nalm-6 or Daudi killing with the addition of anti-CD3. Poor killing of LAK-resistant Nalm-6 or cryopreserved ALL blasts by ALL patients after 17 to 20 days of culture was markedly enhanced in lectin-dependent cellular cytotoxicity (LDCC) assay (Table 4) indicating that the “lytic machinery” for the killing of LAK-resistant targets was present in both cultures. On the other hand, the killing of targets already quite susceptible to effector cell-mediated lysis (eg, K562 or Daudi) in occasional patient samples (eg, ALL patient ML against Nalm-6 in Table 4) was diminished by phytohemagglutinin (PHA).

**DISCUSSION**

The differences in relapse rates after autologous, syngenic, or t-depleted BMT versus allogeneic BMT suggest that activated T cells in the graft may in some cases effectively eliminate residual leukemia. Nevertheless, because almost half of the patients receiving allogeneic BMT for poor-prognosis ALL also relapse, improved in vivo preparative regimens are needed to significantly lower relapse rate. The use of IL-2 in vitro and in vivo has been investigated as a means to amplify a graft-versus-leukemia effect and/or induce LAK activity in marrow against malignancy. Studies with NK cell-resistant dissemi-
nated tumors have shown potent graft antitumor effects using transplantation of IL-2-activated syngeneic bone marrow. Human bone marrow samples incubated with IL-2 for 1 to 3 days have been shown to be superior to peripheral blood IL-2–activated cells in the killing of K562, CEM, and Daudi cell lines as well as fresh lymphoid leukemia blasts. Such an approach, using IL-2–activated spleen cells of rats, was shown to be effective in purging a variety of hematologic malignancies in vitro and to be an effective treatment in vivo against AML.

Because PBMC of BMT patients proliferate poorly in response to IL-2 or anti-CD3 plus IL-2, one technique for post-BMT therapy might use autologous adoptive killer cells from cryopreserved marrow samples obtained before BMT. Therefore, our study sought to compare proliferation and antileukemia cytolytic activity of IL-2–activated cells from marrow versus peripheral blood. Similar proliferation was observed using cellular material from marrow compared with blood with either IL-2–activation or anti-CD3 plus IL-2–activation schemes. Proliferative capacity was significantly increased using the anti-CD3+IL-2–activation scheme compared with IL-2 alone ($P < .01$ for all comparisons, Table 1).

Because in vitro cytotoxicity against hematopoietic tumor
targets may reflect the in vivo lytic potential, cytotoxicity of IL-2– and anti-CD3+IL-2–activated effectors were tested against K562 (NK-sensitive), Daudi (NK-resistant, LAK-sensitive), and Nalm-6 (NK- and LAK-resistant) and cryopreserved GS B-lineage ALL blasts (NK- and LAK-resistant). Patterns of cytotoxicity including studies with anti-CD3 or PHA in the assay indicate that the IL-2– and the anti-CD3+IL-2–activated cultures can have substantial biologic differences in patterns of tumor target lysis. Increased cytotoxicity against the LAK-resistant GS ALL blasts and Nalm-6 in the LDCC assay (Table 4) may be caused by stabilization of effectortarget interaction by PHA; on the other hand, decreased cytotoxicity of the more easily killed targets K562 and Daudi in the presence of PHA may be a function of difficulty in recycling and repetitive killing by lymphoid effector cells. Studies by Stohl et al\(^{20}\) previously demonstrated that anti-CD3 augmentation of cytolytic activity against Daudi targets has IL-2–dependent and IL-2–independent components. In our study, in the presence of IL-2, different patterns of dose-dependent augmentation of killing of Daudi and Nalm-6 by anti-CD3 could be observed (Fig 5). These data may indicate that initial stimulation of these cultures with anti-CD3 in the presence of IL-2 can possibly induce a differential capacity for subsequent signaling through the TCR/CD3 complex than culture in IL-2 only.

Phenotypic analysis of surface CD54 by fluorescence-activated cell sorter (FACS) demonstrated that not only K562 and Daudi have high expression of CD54 as previously reported,\(^{28}\) but also that Nalm-6 had a high density of this target ligand for CD11a/CD18 (P.M.A., D.H., July 1991, data not shown). Nevertheless, killing of Nalm-6 was difficult using either IL-2– or anti-CD3+IL-2–activated cultures. It is possible that B-lineage malignancy cells in vivo may be more analogous to killing of Nalm-6 or cryopreserved autologous ALL blasts (Table 4) than K562 or Daudi cytosis. Because LDCC uses PHA-P in the assay essentially to provide a molecular “glue” between effectors and targets via stable binding of oligosaccharides, the LDCC assay provides an estimate of killing potential regardless of adhesion molecules that were involved in the absence of lectin. Approaches that can potentially bind targets and effectors together in vivo, such as the use of heterofunctional antibodies\(^{28-33}\) or fusion proteins between antibodies and tumor or T-cell–binding ligands, may be analogous to the LDCC assay. If so, the efficacy of anti-CD3+IL-2–activated cells may be enhanced using bispecific antibodies or fusion proteins.

The use of anti-CD3 in combination with IL-2 has been previously shown to lead to very rapid expansion of activated cell numbers using murine splenocytes,\(^{36-39}\) and human PBMC.\(^{40-42}\) Recently it has been shown in a murine model system that in vivo antitumor effectiveness is mediated by CD8–positive cells in collaboration with CD4–positive cells.\(^{43}\) Furthermore, IL-2–activated marrow with IL-2 after BMT in a murine AML model increased survival compared with BMT or BMT+IL-2.\(^{44}\) In view of the former and the potential of brief in vitro anti-CD3 activation followed by in vivo IL-2 administration to yield activated T cells to mediate a graft-versus-leukemia effect, we have examined the potential of cryopreserved marrow cells to respond to anti-CD3+IL-2 activation. In a manner similar to blood,\(^{32-35}\) cryopreserved marrow–derived mononuclear cells responded to IL-2+anti-CD3 with both enhanced proliferation in a dose-dependent synergistic manner and the development of cytotoxicity against hematologic malignancies. Thus, not only IL-2 but also anti-CD3+IL-2 activation strategies may be useful adjuncts to current radiation and chemotherapy preparative BMT regimens in the treatment of resistant hematologic malignancy.

**ACKNOWLEDGMENT**

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Table 4. LDCC of LAK-Resistant Targets With IL-2 and Anti-CD3 + IL-2–Activated Effectors

<table>
<thead>
<tr>
<th>Effector from ALL patient AT on day 17 of culture</th>
<th>Nalm-6</th>
<th>GS ALL Blasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA in assay</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PBMC IL-2 only</td>
<td>4.8 (3.4)</td>
<td>32.9 (9.8)</td>
</tr>
<tr>
<td>PBMC IL-2 + anti-CD3</td>
<td>4.7 (6.5)</td>
<td>53.4 (8.7)</td>
</tr>
<tr>
<td>MMC IL-2 only</td>
<td>2.6 (1.3)</td>
<td>20.6 (3.1)</td>
</tr>
<tr>
<td>MMC IL-2 + anti-CD3</td>
<td>6.3 (4.7)</td>
<td>56.0 (11.8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Effector from ALL patient ML on day 18 of culture</th>
<th>Nalm-6</th>
<th>GS ALL Blasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA in assay</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PBMC IL-2 only</td>
<td>2.6 (2.1)</td>
<td>11.6 (4.4)</td>
</tr>
<tr>
<td>PBMC IL-2 + anti-CD3</td>
<td>35.6 (4.8)</td>
<td>30.9 (3.8)</td>
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<table>
<thead>
<tr>
<th>Effector from ALL patient GS on day 18 of culture</th>
<th>GS ALL Blasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA in assay</td>
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<tr>
<td>PBMC IL-2 + anti-CD3</td>
<td>35.6 (4.8)</td>
</tr>
</tbody>
</table>

Values in the table represent percent cytotoxicity (SD) at E:T ratio of 30:1. Abbreviation: ND, not determined.
REFERENCES


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