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Proliferation Response of Leukemic Cells to CD70 Ligation Oscillates with Recurrent Remission and Relapse in a Low-Grade Lymphoma

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Interactions between TNFR family members and their ligands control lymphocyte death, survival, proliferation, and differentiation (1, 2). CD27 and its ligand CD70 are members of the TNFR family and TNF superfamily, respectively. Tightly regulated expression of CD70 and CD27 on normal lymphocytes ensures the transient availability of these costimulatory signals. CD70 is induced transiently upon activation of B cells (3) and T cells (4). CD27 is absent from immature and mature B cells but is found on memory B cells (5, 6), T cells (7), and NK cells (8). Differentiation to effector T cells is associated to loss of CD27 (9). Cell surface CD27 can be proteolytically cleaved to produce a 32-kDa soluble CD27 (sCD27)4 molecule, which can be detected in the serum and urine (10). Elevated serum sCD27 levels are seen in a number of infectious and autoimmune disease states and are believed to be a marker of T cell activation. Also, various types of B cell malignancies express the CD27 molecule and elevated serum levels of sCD27. There appeared to be a correlation between tumor load and sCD27 levels (11). Studies on the biological role of this receptor ligand pair have mainly addressed the ability of CD27 to regulate the immune response. CD27 signaling appears to play a costimulatory role promoting T cell expansion and differentiation (7, 12–15), as well as enhancement of NK cell activity (16). CD70 has been generally envisaged as a passive element merely required for specific CD27 engagement. Yet, reverse signaling of CD70 was reported. Thus, anti-CD70 mAbs were observed to induce proliferation in a subset of B cell chronic lymphocytic leukemia (CLL) (17) and stimulate NK and γδ T cell-mediated redirected killing (18), suggesting that CD70 might be a signaling molecule. Recently Arens et al. (19) showed that CD70 has reverse signaling properties in murine B cells, initiating a signaling cascade that regulates expansion and differentiation. In contrast to the tightly regulated expression of CD70 on normal cells, constitutive expression of CD70 is found on several human leukemias and lymphomas (17, 20, 21). The finding that cross-linking of CD70 on malignant B cells augments proliferation (17) raised the possibility that CD70 might contribute to progression of these B cell malignancies.

We have reported previously a case of CD5-positive B cell lymphoma, diagnosed as atypical CLL/SLL, which is characterized by recurrent acute waves of active disease (22, 23). The patient experienced within 8 years 27 cycles of leukemic waves followed by spontaneous partial remissions. The first week of the wave characterized by fever, generalized lymphadenopathy, and increasing number of clonal IgM-positive leukemic cells usually >60,000 cells/mm3. After a few days, the symptoms of the acute illness disappeared. Following 2–3 wk, most of the transformed leukemic cells disappeared, and the white blood cell count returned to almost a normal level. Nonetheless, 10–60% of the peripheral lymphocytes were monoclonal B cells. The remission periods lasted 2–7 mo (22). Transient bouts of serum TNF-α and IL-10 preceded the acute phases, which were
characterized by coexistence of CD40L+ T lymphocytes and lymphoma cells in the bone marrow (23). During remissions, residual IgM+ leukemic cells exhibited resting phenotype, low proliferative response to CD40L, and delayed apoptosis in culture. In contrast, the acute phase counterparts were phenotypically activated, underwent rapid apoptosis, and proliferated extensively in response to membrane-anchored CD40L (23, 24).

The existence of a clonal leukemic cell population throughout the course of the disease (22) provides a unique opportunity to characterize molecular changes in the neoplastic cells associated with recurrent activation and regression of the disease. Microarray analysis of the leukemic cells done in this study showed consistent pattern of gene expression characterizing different stages of the disease. Because interactions between CD70 and CD27 provide costimulatory signal in B cell activation, we examined their involvement in progression of this “cyclic” lymphoma/leukemia. Indeed, CD70 and CD27 were among the genes whose expression transiently increased simultaneously with initiation of the acute phase. The leukemic cells from remission periods, but not those from acute phases, responded to CD70 ligation by increased proliferation response to PMA. The results are consistent with transition of cell surface CD70 from responsive to nonresponsive state that occurs concomitantly with initiation of the attack and is coupled to activation and blast transformation of the leukemic cells.

Materials and Methods
Isolation of leukemic cells and determination of sCD27 in the serum

PBMCs were separated from heparinized blood by Ficoll (Nycomed Pharma AS) density gradient centrifugation and stored in liquid nitrogen. Where indicated the patient’s leukemic cells were purified from PBMCs on CD19 magnetic beads (Dynal), according to the manufacturer’s instructions. The purified cells contained 94–98% CD19+CD5+ double-positive leukemic cells. Viability of the leukemic cells, examined by trypan blue exclusion, was always leukemic cells. Viability of the leukemic cells, examined by trypan blue exclusion, was always 94–98%. The patient’s PBMCs or CD19+ purified leukemic cells were cultured at 2 × 10^7/ml in RPMI 1640 supplemented with 10% FCS and 50 μg/ml gentamicin sulfate (conditioned medium (CM)), at 37°C in 5% CO₂.

Flow cytometry

For comparison of cell surface marker expression, cells that had been cryopreserved during different disease phases were thawed, processed, and analyzed simultaneously. A total of 4 × 10^5 PBMCs was incubated in 0.4 ml of RPMI 1640 containing 10% FCS for 20 min at 20°C with CD19 PerCP, CD5 APC, CD27 R-PE, and CD70 FITC, washed in PBS, and analyzed on dual laser FACS Calibur flow cytometer from BD Biosciences. Discrimination of leukemic cells from the normal hematopoietic cells was done by gating on cells expressing CD19 and CD5. CD70 and CD27 expression on the leukemic cells was scored within the gated CD19+CD5+ cells. The following mAbs were used for four-color FACS analysis: R-PE-labeled CD27 clone M-T271 and FITC-labeled CD70 clone (clone Ki-24) (BD Biosciences Pharmingen), allopurinol, Apo2.7 and CD5 (clone L17F12), and PerCP-labeled CD19 (BD Biosciences).

Tests of apoptosis and cell proliferation

The patient’s PBMCs or CD19+ purified leukemic cells were cultured at 2 × 10^7/ml in RPMI 1640 supplemented with 10% FCS, and 50 μg/ml gentamicin sulfate (conditioned medium (CM)), at 37°C in 5% CO₂. Spontaneous apoptosis of the leukemic cells in culture was examined by Annexin V FITC staining. A total of 4 × 10^5 leukemic cells was cultured in 96-well flat-bottom plates in CM in presence or in absence of 1–9 μg/ml anti-CD70 mAb (clone Ki-24; BD Pharmingen) or anti-CD27 mAb (clone 1A4; Immunotech). Apoptosis of the cultured leukemic cells was determined following 7 or 24 h incubation in CM at 37°C in 5% CO₂. The cells were washed and resuspended in a buffer containing 10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂, Annexin V FITC (1.50) (Roche) and propidium iodide (50 μg/ml) (Sigma-Aldrich) were added, and the cells were stained for 20 min at 20°C and immediately assessed on a Beckman Coulter Epics MCL flow cytometer. Proliferation of the leukemic cells was tested by [³H]thymidine uptake. A total of 2 × 10^3 cells was cultured in 96-well plates in CM in triplicates. To examine the effect of the Abs on proliferation, the leukemic cells were first allowed to bind the Abs in preincubation at 20°C for 30 min with 1–9 μg/ml azide-free CD70 mAb (clone Ki-24) or 1–9 μg/ml azide-free CD27 mAb (clone 1A4), followed by addition of 1 ng/ml PMA (Sigma-Aldrich). To examine the proliferative response of the leukemic cells to CD40L, purified leukemic cells were cultured with gamma-irradiated CD40L-presenting L cell, as detailed previously (23). The cells were incubated at 37°C in 5% CO₂ for 4 days. During the last 18 h of the culture, 1 μCi [³H]thymidine (20 Ci/mmol; PerkinElmer) was added to measure proliferation. All apoptosis and proliferation assays were repeated three times. The results of the microarray analysis representing all probe sets (pivot for supp) and those representing the 216 probe sets that were up-regulated upon initiation of the acute phase (216 subcluster gene list) are accessible in the online supplemental material.5

Results

Transcriptional profiling of the leukemic cells during remission and the acute phase

The recurrent leukemic attacks enabled us to search for changes occurring consistently in the leukemic cells during the spontaneous induction and regression of the acute phase. Thus, initiation of the attacks was indicated by high fever followed by a burst of blast-like lymphocytes in the blood peaking on the third day and decreasing spontaneously within 2–3 wk. During remission periods 10–60% of the mononuclear cells were the clonal IgM, CD5+CD19+ leukemic cells (22), cDNA microarrays, representative of ~12,000 genes, were used to compare transcriptional profiles of the leukemic cells during remission periods and on days 3, 5, 7, and 11 of the attack. The leukemic cells were purified by magnetic cell separation of CD19+ cells. Expression of hundreds of genes correlated with that of the leukemic phase. Furthermore, each stage of the attack was characterized by a different expression profile (Fig. 1A). Fig. 1B depicts a cluster of 216 genes, including CD70 and CD27, which were up-regulated during transition from remission to the third day of the leukemic attack. This cluster of stimulated genes includes, among others, 16 genes related to immune response, 14 related to cell adhesion, 21 related to

5 The online version of this article contains supplemental material.
and CD27 and investigate their possible involvement in the induction and regression of the recurrent leukemic attacks.

Expression of CD70 and CD27 oscillates in correlation with the leukemic phase

The expression level of CD70 and CD27 in the purified leukemic cells was verified by RTQ-PCR analysis. In an attempt to obtain expression ratios that more reliably reflect the transition from remission to acute phase, leukemic cells from three different remission periods were used as reference for comparison of gene expression during the attack. Three days following the burst of the attack the level of CD70 and CD27 increased 38- and 25-fold, respectively. During the next 8 days, in parallel with the spontaneous decay of the attack, expression of CD70 and CD27 gradually decreased approaching the remission phase level (Fig. 2). The higher folds of expression compared with those indicated in Table I (7 and 2.6, respectively) probably reflect the use of a different remission cell reference. Higher folds of expression were also found in this assay for TGFβRIII and BIK (240 and 150 vs 42.2 and 32.0, respectively). The time of maximal expression of TGFβRIII and BIK remained the fifth day of the attack (data not shown).

Cell surface expression of CD70 and CD27 was determined on CD19-CD5+ leukemic cells by four-color FACS analysis. Flow cytometric dot plots show that expression of CD70 and CD27 on the majority of the leukemic cells increased upon induction of the attack (Fig. 3A). The cells were examined during four different remission periods, and five of nine leukemic attacks occurred during the first 3 years of the disease. CD70 was expressed on the majority of CD19-CD5+ leukemic cells during the attack but on <10% of them during remission periods, while CD27 was consistently expressed on all the leukemic cells. The level of both CD70 and CD27 expression was maximal on the second to third day of the attack (Fig. 3B). Thus, during the peak of the acute phase, 80% of the leukemic cells coexpressed high levels of membrane CD70 and CD27 while during remission periods 90% of the leukemic cells expressed CD27 but no detectable CD70 and 5–10% of them coexpressed CD70 and CD27. The intensity of CD70 staining during remission indicates that the small fraction of leukemic cells included in the CD70-positive subpopulation express high levels of CD70. This oscillation pattern of membrane CD70 and CD27 expression was consistent during the first 3 years of the disease while the patient did not receive any specific medication. Later on, following the ninth leukemic attack, prednisone at 30 mg/day was instituted at the onset of the attack, and the dose was subsequently tapered off over a period of 30 days. The stimulation of membrane CD70 expression during the attack was inhibited by the prednisone treatment while elevated expression of membrane CD27 was stabilized (Fig. 4).

sCD27 in the patient’s serum

sCD27, which is capable of binding cell surface CD70, may impede the cellular response of membrane CD27 to CD70 presenting cells (25). Hence, examination of the involvement of CD70-CD27 interaction in progression of the disease requires determination of sCD27 levels in the patient’s serum during different phases of the disease. The concentration of sCD27 was determined by ELISA and was expressed as units per milliliter in reference to sera of 11 healthy blood donors. The concentration of sCD27 in the sera of normal donors was 185 ± 80 U/ml. The patient’s sera were examined, while prednisone treatment was given during the attack because no sera were available before this period. The patient’s sera were taken on the
sixth or seventh day of four different attacks, during a regression period (day 20 of the attack), and during three remission periods (30, 90 and 150 days from initiation of the recent attack). During the sixth and seventh day of the attack the levels of sCD27 were 1600 – 3500 U/ml. These levels decreased to 600 U/ml during regression of the attack in the next 2 wk and remained at the same level during remission periods (Fig. 5). Thus, the level of sCD27 in the patient’s serum oscillates with the leukemic cycle being 3- to 5-fold of normal level during remission periods and further increased 3- to 5-fold during the attacks.

Functional analysis of CD70 and CD27 ligation on the leukemic cells

Ligation of CD70 or CD27 on the leukemic cells membrane does not affect the rate of apoptosis. Extreme changes in the rate of apoptosis of the leukemic cells during the leukemic cycle (22, 23) and oscillation in expression of some BCL-2 family genes (24) prompted us to examine the possible involvement of membrane CD70 and CD27 in apoptosis. Leukemic cells from acute phases and remission periods were incubated with 1–9 μg/ml mAb directed to CD70 or CD27, (K1-24 or 1A4, respectively).

Functional analysis of CD70 and CD27 gene expression during remission and the leukemic attack

<table>
<thead>
<tr>
<th>Functional Category/Gene Name</th>
<th>GenBank Accession No.</th>
<th>Fold Change Relative to Remission Day in the Attack</th>
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<tr>
<td>Cell activation and signal transduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD80 Ag</td>
<td>M27533</td>
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</tr>
<tr>
<td>Pre-B cell colony-enhancing factor, PBEF1</td>
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<tr>
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</tr>
<tr>
<td>CD137 ligand, TNFSF9</td>
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<tr>
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<tr>
<td>CD27, TNFRSF7</td>
<td>M63928</td>
<td>2.6</td>
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FIGURE 2. RTQ-PCR analysis of CD70 and CD27 gene expression in the leukemic cells during remission and the leukemic attack. Comparative ratio analysis by RTQ-PCR of CD70 (TNFSF7) and CD27 (TNFRSF7) gene expression in purified leukemic cells during the leukemic cycle. The y-axis shows relative gene expression. The average expression level of CD70 and CD27 during three different remission periods (R) was taken as 1. Expression level of CD70 and CD27 increased 37- and 25-fold, respectively, during transition from remission to the third day of the attack followed by gradual decrease to the remission phase level.

FIGURE 3. Cell surface expression of CD70 and CD27 on the leukemic cells is increased concomitantly with transition from remission to the acute phase. Four-color FACS analysis shows cell surface expression of CD70 and CD27 gated on CD19+/CD5+ double-positive leukemic cells. A, Flow cytometric dot plots illustrate labeling of CD19+CD5+ cells with Abs to CD70 and CD27 during remission and on the third day of the leukemic attack. The percentage of cells in the upper right quadrant (CD70+/CD27+) is indicated. B, Accumulated data represent staining intensity and percentage of CD70+/CD27+ and CD70−/CD27− leukemic cells during early (R1) and late (R2 and R3) remission periods and on different days (2nd to 11th) following the initiation of the leukemic attack. The percentage of CD70−/CD27− cells increased from 5 to 10% during remission periods to 80–90% on the second to third day of the attack. Maximal density of cell surface CD70 and CD27 was observed at an early stage of the attack.
The effect of the Abs on spontaneous apoptosis of the leukemic cells in culture was examined by AnnexinPI analysis. The indicated mAbs were reported previously to affect some lymphocyte activities. Binding of KI-24 anti CD70 mAb to CD70-positive glioblastoma cells increased alloploproliferation and decreased apoptosis of T lymphocytes in a mixed tumor T lymphocyte reaction (25), while 1A4 anti-CD27 mAb inhibited lymphocytes activation and proliferation (26, 27). Fig. 6 shows that the bound CD70 and CD27 mAbs did not affect the rate of apoptosis, either that of the acute phase leukemic cells exhibiting rapid apoptosis or that of the remission phase leukemic cells exhibiting slower apoptosis. This result suggests that cell surface CD70 and CD27 are not directly involved in apoptosis of the leukemic cells.

Ligation of CD70 enhances proliferation of the leukemic cells from remission but not those from the acute phase. The leukemic cells of this patient do not proliferate in culture spontaneously. CD70 ligation with anti-CD70 mAb strongly enhanced proliferation of the remission phase leukemic cells submitted at the induction of the acute phase and its reduction during remission was attenuated.

Elevated levels of sCD27 in the patient’s serum. Serum samples were examined for sCD27 on the day indicated following the initiation of the attack. Sera representing two different “leukemic cycles” were analyzed. Sera of 11 normal controls contained 185 ± 80 U/ml sCD27. The levels of sCD27 in the patient’s serum were elevated during remission and were further increased during the attack.

Abs to CD70 and CD27 do not affect the rate of apoptosis of the leukemic cells. Spontaneous apoptosis of the leukemic cells in culture was examined. The results expressed by annexin-positive cells show the percentage of apoptotic cells following 7 or 24 h of incubation. Leukemic cells from the peak of the acute phase exhibiting rapid spontaneous apoptosis (39% in 7 h and 94% in 24 h) or leukemic cells from remission period exhibiting slower apoptosis (23% in 7 h and 36% in 24 h) were incubated in the absence or in the presence of 6 μg/ml of either anti CD70 mAb (clone ki-24) or CD27 mAb (clone 1A4). Data are expressed as average values of four different experiments with cells from two different “leukemic cycles.” The rate of spontaneous apoptosis of the leukemic cells was not affected by either mAb.

Discussion
In this study, we have investigated a possible contribution of CD70 and CD27 to progression of an unusual B cell lymphoma/leukemia characterized by cycles of leukemic phase alternating with spontaneous remission. We focused here on CD70 and CD27 expression.
CD70 mAb enhances proliferation of purified leukemic cells obtained during remission, whereas CD40L enhances proliferation of those obtained during the attack. The leukemic cells were taken on the third and fifth day of the acute phase and during two different remission periods and purified on CD19 beads. The purified leukemic cells were cultured in the absence or in the presence of PMA (1 ng/ml) with or without 5 μg/ml of either anti CD70 mAb (clone ki-24) or CD27 mAb (clone 1A4). To examine the effect of CD40 triggering, the leukemic cells were cultured with gamma-irradiated CD40L-presenting cells. Shown is one of three representative experiments. CD70 mAb enhanced proliferation of the leukemic cells obtained during remission and did not affect the acute phase cells. CD40L induced proliferation of the leukemic cells obtained during the attack and did not affect the remission phase cells.

CD27 because the interaction between them may affect lymphocyte activation, proliferation, and death (1, 2), raising the possibility that they are involved in the induction and regression of the recurrent acute attacks. The results presented herein were mostly obtained during the first 3 years of the disease during which the patient experienced nine leukemic cycles without any specific therapy. However, cell surface expression of CD70 and CD27 and sCD27 in the serum were also examined during a later stage of the disease while the patient received prednisone during the attack. Transcriptional profiling of the leukemic cells by microarray analysis showed massive changes in gene expression between different stages of the disease. Increased expression of cell growth enhancers and inhibitors, as well as apoptosis enhancers, was observed at an early stage of the leukemic attack. For example, initiation of the attack was followed by highly increased expression of galecin 1, implicated in enhancements of the Ras signal transduced to ERK (28) via the MEK pathway and protein kinase B activity (29) via the PI3K pathway. Relevant to this may be the observation of Arens et al. (19) who have reported that CD70 signaling in murine B cells enhances cell growth, the elevated expression of TGF

\[
\text{CDK2AP1}, \text{which is induced by TGF}\]

functions in CLL as an autoreactive cytokine circuit regulating the malignant cells proliferation. Alternatively, the T cells infiltrating the lymphoid stroma in this patient (23) could as well provide the CD27 as ligand for CD70 on the tumor cells.

We have previously found that the patient’s cells exhibited resting state during remission periods and activated state at the initiation of the leukemic attack. Cell activation became apparent by blast transformation and elevated expression of CD23, CD25, ICAM-1, and LFA-3 (22, 23). The cell activation, the bouts of TNF-α and IL-10 detected in the serum during early acute phases, and the production of TNF-α and IL-10 by activated leukemic cells in culture (23) suggested that the neoplastic cells responded to an activation signal at the onset of each leukemic attack. The leukemic cells from the attack but not those from remission exhibited high proliferative response to CD40L. The potent responsiveness of the acute phase leukemic cells to CD40L is compatible with the finding that during acute phase the patient’s bone marrow was heavily infiltrated with both CD40L-positive T cells and the
neoplastic cells (23). These results suggested that CD40L-/-reactive T lymphocytes residing in the patient’s bone marrow support proliferation of the neoplastic cells during the acute leukemic phases. Notably, however, the proliferation response of the resting, remission phase cells to CD40L was significantly lower than that of activated, acute phase cells (23). This observation implied that initiation of extensive proliferation of the resting neoplastic cells at onset of acute phases requires an additional activation signal. The finding herein that the remission phase leukemic cells proliferate effectively in response to CD70 ligation and less effectively in response to CD40L while the acute phase cells do not respond to CD70 ligation but proliferate in response to CD40L suggests that signaling via CD70 precedes that of CD40 in promoting the leukemic attack.

The recurrent change in CD70 responsiveness of the leukemic cells could be explained by several ways: 1) The CD70 unresponsiveness of the acute phase cells could result from binding of sCD27 to cell surface CD70 making it unavailable to the stimulating CD70 Ab. However, this possibility seems unlikely because the ratio of sCD27 to membrane CD70 is three to five times higher during remission periods while the cells respond effectively to CD70 signaling. 2) The second interpretation is based on a previous observation (22) that two B cell clones with differently rearranged H chain genes consistently appeared in the blood of this patient during recurrent acute phases. During the spontaneous remissions, one clone (termed the “acute clone”) regressed while the second clone (the “remission clone”) remained relatively stable. Thus, the two clones of the leukemic cells may differ in their CD70 responsiveness as follows: the remission clone, which comprises the majority of leukemic cells during remission, responds to CD70 ligation by enhanced proliferation, while the second clone, which dominates the acute phase, does not respond to CD70. However, preferential proliferation of the remission clone cells contradicts the previous results, which demonstrated that proliferation and death of the acute clone cells and not that of the remission clone cells determine the cyclic nature of the disease (22). 3) The overall results suggest that the leukemic cells of the acute clone and not those of the remission clone consistently express cell surface CD70 and respond to its ligation during remission but not during the attack. We do not know what may cause this switch between responsive and nonresponsive state of cell surface CD70. A possible explanation will be a change in the signaling cascade. An alternative explanation could be a conformational change of membrane CD70 affecting its responsiveness. A structural rearrangement of cell surface integrins resulting in change of their ligand binding activity was described previously (34).

In conclusion, we have found that in this lymphoma/leukemia case a switch from responsiveness to unresponsiveness of CD70 occurs on the leukemic cells during initiation of the acute phase. Before this transition, cell surface CD70 could supply a costimulatory receptor for cell proliferation and thus provide a growth advantage to the responder leukemic clone. Termination of this CD70 response concurrently with cell activation and blast transformation of the leukemic cells could contribute to regression of the acute phase in this “cyclic” disease. The finding that spontaneous remission follows the acute phase despite elevated proliferation response to CD40 ligation supports the notion that continuous growth of the leukemic cells requires CD70 response in this case. In human leukemias and lymphomas that constitutively express cell surface CD70, it may provide a costimulator receptor for tumor cells proliferation. Whether this responsiveness is required for tumor cell growth and could be regulated by a switch in CD70 reactivity should be examined in a future study.

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Disclosures

The authors have no financial conflict of interest.

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