Resistance to FasL and tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in Sézary syndrome T-cells associated with impaired death receptor and FLICE-inhibitory protein expression

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Because of the low proliferative potential of tumor cells in patients with Sézary syndrome (SzS), their accumulation has been suggested to be due to defective regulation of apoptosis. We analyzed the sensitivity to soluble Fas-ligand (FasL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), 2 members of the TNF superfamily in peripheral blood leukocytes (PBL) from patients with SzS. Compared with healthy donors, CD4+ cells from patients with SzS were completely resistant to FasL in 9 of 16 cases. Of these 9 FasL-resistant cases, 4 revealed a loss in Fas (CD95) expression, whereas the remaining 5 exhibited normal or enhanced Fas expression. In the latter 5 cases, the apoptosis inhibitor cFLIP was overexpressed in CD4+/CD26− tumor cells compared with CD4+/CD26+ cells from FasL-sensitive patients and healthy donors. Furthermore, resistance to TRAIL receptor 2 were observed in 16 of 16 SzS PBLs. It is noteworthy that resistance to FasL could be overcome by the use of a hexameric FasL or upon exposure of SzS cells to interferon-α (IFN-α) or IFN-γ, the latter by an increase of Fas expression. Our data on primary SzS lymphocytes reveal frequent resistance to apoptosis induced by FasL and TRAIL, which may contribute to their accumulation in patients with SzS and be relevant at a therapeutic level. (Blood. 2008;111:4780-4787)

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Table 1. Characteristics of the SzS patients included in the study

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Percentage of SzS cells was determined by flow cytometry or immunostaining of CD4+CD26- cells in peripheral blood after cytopsin. Ratios are calculated as follows: number of CD8/mL divided by number of CD4/mL of blood. CD4+ cells from patients with SzS were considered sensitive to Fasl for <60% annexin-V-FITC expression by flow cytometry. Fas was considered to be significantly expressed when the mean fluorescence intensity (MFI) was >2 in CD4+ SzS cells. ND indicates not determined; –, negative; +, positive/moderate; and ++, positive/high.

Methods

Patient material and cell lines

Peripheral blood leukocytes (PBL) from patients with SzS were obtained after Ficoll isolation and frozen in fetal calf serum and 10% dimethyl sulfoxide until use. All the experiments described in the present work were performed with frozen PBL from patients with SzS diagnosed accordingly to ISCL criteria. Tumor burden (reported in Table 1) is defined as the percentage of circulating SzS cells and determined on sections of formalin-fixed peripheral blood buffy coats. T-cell receptor (TCR) rearrangement was positive for all the patients with SzS included in the present study. CD4/CD8 ratios are reported for each patient in Table 1. Donation of blood by patients or healthy volunteers in this study conformed to University of California, San Diego, institutional review board approval, and informed consents were obtained in accordance with the Declaration of Helsinki. The SzS cell line HUT78 was from the American Type Culture Collection (Manassas, VA). The MF cell line MyLa and the SzS cell line SeAx were kindly given by Prof K. Kaltoft and Prof H. Bachelez (Inserm, Hôpital Saint-Louis, Paris, France).

Sorting of CD4+/CD7− or CD4+/CD26− tumor cells

After thawing, CD4+/CD7− or CD4+/CD26− cells from patients with SzS and healthy donors (HD) were magnetically sorted using depletion of CD7+ or CD26+ cells followed by the positive selection of CD4+ cells with an AutoMacs (Miltenyi Biotech, Bergisch Gladbach, Germany). For the depletion of CD7+ or CD26+ cells, total PBL were stained with a phycoerythrin-conjugated anti-CD7 (clone M-T701; BD Biosciences, San Jose, CA) or anti-CD26 antibody (clone M-A261) and anti-phycoerythrin-conjugated beads (Miltenyi Biotech). The CD26- fraction was collected, labeled with anti-CD4-conjugated beads, and the CD4+ fraction was collected.

Analysis of the expression of death receptors

PBL from patients with SzS and HD were stained with monoclonal antibodies to CD4 (clone RPA-T4) and CD7 (clone M-T701) or CD26 (clone M-A261; all from BD Pharmingen), and monoclonal antibodies to Fas, TRAIL-receptor 1 (DR4, clone HS101), 2 (DR5, clone HS201), 3 (DrR1, clone HS301), and 4 (DrR2, clone HS402; Apotech, Lausen, Switzerland). Flow cytometric analysis was performed on a FACSScan with the CellQuest Software (BD Biosciences, Allschwil, Switzerland).

Induction and analysis of apoptosis

Fresh (CTCL cell lines) or thawed (SzS and HD) cells were incubated at 106/mL in 96-well plates in 200 μL of RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and antibiotics. SzS and HD CD4+ cells or CTCL cell lines were treated with increasing doses of soluble recombinant Fasl (SuperFasLigand; Apotech), MegaFasLigand (kindly provided by Apoasis S.A./Topotarget Switzerland, Lausanne, Switzerland) or soluble recombinant TRAIL (SuperKillerTRAIL; Apotech) for 6 to 8 hours. Early apoptosis of the treated cells was analyzed by flow cytometry after staining with annexin-V (BD Pharmingen) and propidium iodide (Sigma-Aldrich, St Louis, MO). When indicated, cells were treated overnight with 100 IU/mL recombinant IFN-α or IFN-γ (Peprotech, London, United Kingdom) before treatment with SuperFasLigand.

Quantitative reverse transcription polymerase chain reaction

Total RNA were extracted with a QIAgen Mini Kit upon manufacturer’s instructions and subjected to DNase I (Qiagen, Basel, Switzerland) treatment. Two micrograms of RNA were converted into cDNA using SuperScript II (Qiagen). The expression of cFLIP in purified CD4+CD26− cells was compared with 3 stably expressed housekeeping genes, namely HsEEF1A1, GusB, and ALAS. The primers used for cFLIP cDNA amplification were CCGAAAGTACAAG-CAGTCGTGTTCAA (forward) and TTGGATGCTGCTTGAGAA (reverse).

Statistical analysis

When indicated, unpaired Student t test was applied. Differences were considered significant for P values less than 0.05.

Results

Heterogeneous FasL sensitivity in cutaneous lymphoma cell lines and circulating CD4+ cells from patients with SzS

Upon analyzing the sensitivity of a large number of cell lines to Fasl, we could show that 1 of 3 CTCL cell lines was resistant to apoptosis when exposed in vitro to a hexameric form of soluble Fasl. (SuperFasL) that has previously been shown to be a potent inducer of Fas-mediated apoptosis in Fas expressing cell lines (Figure 1C and data not shown). As recently shown by Braun et al.,23 we observed, with the use of a different apoptosis assay, that in the FasL-resistant SeAx cell line, the loss of sensitivity to soluble Fasl correlated with a loss of Fas surface expression compared with the 2 other cell lines (HUT78 and MyLa; Figure 1C).
To understand the cause of the above observed resistance to soluble FasL in CD4⁺ cells from patients with SzS, we analyzed the expression of Fas on CD4⁺ cells from HD and patients with SzS. In HD, Fas expression on CD4⁺ cells was quite strong and varied little from donor to donor (mean fluorescence intensity [MFI] = 4.9 ± 1.09) (Figure 1A). This was not the case in patients with SzS, in which Fas expression on CD4⁺ cells varied considerably among patients. Four of 16 (25%) patients with SzS (patients 1, 2, 6, and 8), exhibited a decreased level of Fas expression on CD4⁺ cells (mean MFI = 1.7) compared with HD (mean MFI = 4.75). The remaining 12 of 16 patients (75%) revealed higher levels of Fas expression on CD4⁺ cells than HD (mean MFI = 8.2) levels of Fas expression on CD4⁺ cells compared with HD. Because tumor cells comprise only a fraction of the total CD4⁺ cells, analysis of Fas expression was also specifically analyzed on CD4⁺/CD7⁻/CD26⁻ subpopulations, indicating that the observed loss of Fas expression on CD4⁺ cells in patients with SzS is not selective for the tumor population of CD4⁺ cells (data not shown).

To analyze the sensitivity of circulating CD4⁺ cells from patients with SzS and HD to FasL, fresh or thawed PBL were exposed in vitro to soluble FasL (SuperFasL), and apoptosis was assessed by annexin-V/PI staining. In the 10 HD tested, FasL induced apoptosis of CD4⁺ cells in a dose-dependent manner, with an average of 50% apoptosis (46.5 ± 6.5% [SD]) occurring after 8-hour exposure to 100 ng/mL of soluble FasL (Figure 1A). In patients with SzS CD4⁺ cells, the degree of apoptosis induced by soluble FasL under the same conditions varied significantly among
Within the 16 patients with SzS tested, CD4+ cells from 9 of 16 patients with SzS (56%, patients 1-9) were significantly (P < .05) more resistant to soluble FasL-mediated apoptosis (90 ± 6.9% viable cells) than HD CD4+ cells (Figure 1A, Table 1). In the remaining 7 patients with SzS (44%, patients 10-16) sensitivity to FasL was similar to that of HD (mean 46.8 ± 11.3% apoptotic cells) (Figure 1A and Table 1). Furthermore, a certain degree of correlation (R² = 0.54) between FasL-resistance and tumor burden was observed (Figure 1B). It is noteworthy that in patients with a high tumor burden (> 80% circulating SzS cells) FasL was not able to induce apoptosis of CD4+ cells (89.5% ± 15% viable CD4+ cells), whereas an average of 50% CD4+ cell apoptosis (51.6 ± 17.3%) was observed in patients with low or medium (< 50% circulating SzS cells) tumor burden (Figure 1B, Table 1).

In 4 of 16 (25%) patients with SzS, the significantly reduced Fas expression on CD4+ cells was correlated with significant resistance to FasL-induced apoptosis (patients 1, 2, 6, and 8; Figure 1A). In 7 of 16 (43.75%) patients with SzS, CD4+ Fas expression correlated well with sensitivity to FasL-induced apoptosis (patients 10-16). Finally, in the remaining 5 of 16 (31.25%) patients with SzS, significant resistance to FasL-induced apoptosis was observed despite CD4+ cell Fas expression levels similar or higher than that observed in HD (patients 3, 4, 5, 7, and 9). The above observations indicate that decreased Fas expression on SzS CD4+ cells was not the sole mechanism responsible for resistance to soluble FasL.

**Overexpression of the cFLIP gene in circulating tumor cells from patients with SzS**

The observation that 5 of 16 patients with SzS (patients 3, 4, 5, 7, and 9) CD4+ cells were resistant to soluble FasL-induced apoptosis, despite significant Fas expression, suggests that molecular mechanisms other than loss of Fas-expression may account for the observed apoptosis resistance. To assess whether the intracellular death receptor-mediated apoptosis inhibitor cFLIP could be involved in apoptosis resistance, quantitative cFLIP real-time polymerase chain reaction (RT-PCR) was performed on sorted CD4+/CD26- cells from the 5 patients described above and compared with Fas-expressing FasL-sensitive patients with SzS (n = 4, patients 11, 14, 15, and 16) and HD (n = 5). The magnetically sorted CD4+/CD26- populations exhibited a purity of more than 95% (data not shown). In the 5 tested CD4+/CD26- populations from FasL-resistant patients with SzS, the relative expression of cFLIP was significantly increased compared with CD4+/CD26- cells from FasL-sensitive patients with SzS (1.9 ± 0.26 vs. 0.8 ± 0.17, respectively; P < .05; Figure 2, Table 1) and HD (1.9 ± 0.26 vs. 0.45 ± 0.15, respectively; P < .05; Figure 2, Table 1). The same analysis was performed in the HUT78, MyLa, and SeAx CTCL cell lines, and cFLIP gene expression was found to be similar to that measured in healthy donors (0.47 ± 0.45 vs. 0.45 ± 0.17, respectively; P = not significant; Figure 2). These data suggest that cFLIP overexpression may be associated with resistance to FasL-mediated apoptosis in Fas-expressing CD4+ cells in patients with SzS.

**CD4+ cells from patients with SzS are resistant to TRAIL, and the CD4+CD26- tumor cell population selectively lacks surface TRAIL-R2 (DR5)**

Because soluble TRAIL is in clinical development for the treatment of cancer, we tested the sensitivity of CD4+ cells from HD and patients with SzS to soluble TRAIL. In contrast to CTCL cell lines and, to a lesser extent HD CD4+ cells, which are both shown to be sensitive to soluble TRAIL in a dose-dependent manner, CD4+ cells from all 16 (100%) tested patients with SzS were completely resistant to TRAIL-induced apoptosis in vitro (Figure 3A, Table 1). Furthermore, the observed resistance to TRAIL-induced apoptosis in patients with SzS was independent of tumor burden (Table 1).

To further investigate the cause of resistance to TRAIL, the expression of the death receptors TRAIL-receptors 1 (DR4) and 2 (DR5) and the decoy receptors TRAIL receptors 3 (DcR1) and 4 (DcR2) was investigated by fluorescence-activated cell sorting in PBL from 10 HD and 16 patients with SzS. No difference of expression of DR4 and DcR1 was found between HD and patients with SzS on total CD4+ cells or CD4+ CD26- cells (Figure 3B,C). By contrast, the expression of both TRAIL-receptor 2 (DR5) and DcR2 were found to be significantly decreased in total CD4+ cells from patients with SzS compared with HD (Figure 3B, Table 1). It is noteworthy that the observed loss in TRAIL-receptor 2 (DR5) and DcR2 expression selectively affected the CD4+/CD26- cells from patients with SzS (Figure 3B). Taken together, these data reveal a selective loss of TRAIL-receptor 2 (DR5), the major receptor involved in TRAIL-induced apoptosis, in a tumor-restricted manner.

**A novel hexameric form of FasL (MegaFasL) or preincubation with IFN-α/γ could partially overcome the resistance of SzS CD4+ cells to apoptosis**

CD4+ cells from patients resistant to apoptosis induced by the above agents as well as commercially available soluble FasL (SuperFasL) were exposed to a novel genetically engineered hexameric form of soluble FasL (megaFasL [MFL]). For this experiment, 4 FasL-resistant patients were selected (patients 6, 7, 8, and 9; see Figure 1A, Table 1). By contrast to SuperFasL, MFL was able to induce significant apoptosis in circulating tumor cells from 3 of 4 (75%) of the selected patients (patients 7, 8, and 9). In these patients, the average IC₅₀ after 8 hours in vitro MFL exposure ranged from 3 to 50 ng/mL, as opposed to more than 1 µg/mL with SuperFasL. No apoptosis was observed in patient 6 in whom Fas expression on CD4+ cells was completely absent (Figure 4).

IFN-α has proven clinical efficacy in the treatment of CTCL including SzS. To determine whether IFN can affect the sensitivity to FasL, CD4+ cells from 2 selected patients with SzS with low Fas
expression (patients 1 and 2) and from 2 healthy donors (HD 1 and 2) were exposed in vitro overnight to 100 IU/mL IFN-α or IFN-γ and subsequently exposed to 100 ng/mL of SuperFasL. Exposure to either IFN-α or IFN-γ resulted in a 40% to 70% increase in Fas expression on the surface of CD4+ cells in 2 of 2 HD and 2 of 2 patients with SzS (Figure 4B top panels). When apoptosis was assessed under the same conditions, the observed increased Fas expression upon exposure to IFN-α or IFN-γ was associated with a significant (40%-80%) increase in FasL-mediated apoptosis (Figure 4B bottom panels).

Discussion

In the present work, we demonstrate that in patients with SzS, CD4+ T cells harbor significant defects in death receptor-mediated apoptosis signaling. These defects affect both the FasL and TRAIL signaling pathways and are associated in all cases with either a loss of expression of the cell surface receptor for these 2 ligands, or an increased expression of the gene encoding the intracellular apoptosis inhibitor cFLIP. Furthermore, we show that in the case of Fas, at least, this signaling defect can be circumvented in a potentially therapeutically meaningful manner in vitro either with a genetically engineered hexameric form of FasL or by pre-exposure to IFN-α or IFN-γ.

Appropriate Fas-mediated cell death is essential for adequate control of lymphocyte homeostasis. It is noteworthy that elimination of T cells after encountering a specific antigen or superantigen proceeds via activation-induced cell death, a process that is dependent on appropriate Fas signaling of cell death. Furthermore, in both mice and humans, Fas gene mutations leading to defective Fas/signaling result in lymphoproliferative disorders as a consequence of lymphocyte accumulation.24,25 Because in CTCL, tumor cells have been demonstrated to be long-lived activated CD4+ cells, defects in Fas-mediated apoptosis have been previously proposed based on studies of protein expression, but the functional consequences and detailed analyses of Fas signaling in CTCL is lacking.26 It has recently been shown that apoptosis defects exist in
CTCL cells, but the majority of the reported functional data were obtained with 4 established CTCL cell lines, a setting that does not necessarily adequately reflect the clinical situation. In the present work, we specifically analyzed apoptosis defects in ex vivo primary SzS cells from 16 patients.

Using immunohistochemistry, decreased Fas expression by semiquantitative analysis of anti-Fas labeling of skin-infiltrating CD4 T cells has been reported in tumor-stage MF and CD30 large cutaneous large T-cell lymphoma. Likewise, in 4 and 3 cases of SzS, Dereure et al and Meech et al, respectively, provide data showing that Fas expression is decreased on circulating CD4 or CD45RO-positive cells without further specification of the T-cell phenotype. In the present work on a significant number of patients with SzS, we functionally demonstrate for the first time that, compared with healthy donors, circulating CD4 cells from patients with SzS are completely resistant to soluble FasL in 56% of cases. It is noteworthy that this functional defect in Fas signaling is only accounted for by a loss of Fas expression in 44% of the Fas-resistant cases, and in such cases, the loss of Fas expression was not restricted to CD4+CD26 cells phenotypically consistent with the tumor cell population. The cause of the observed loss of Fas expression on tumoral and nontumoral CD4 T cells of patients with SzS remains to be determined. Fas gene mutations or splice variants have been reported in MF but no data are available for SzS; moreover, the latter would not account for the observed loss of Fas expression on nontumoral CD4 T cells of patients with SzS. A possible explanation that remains to be investigated is that the Th2 cytokine milieu that predominates in SzS may affect Fas expression and/or susceptibility to Fas-mediated apoptosis in CD4+ cells. In support of this, in a recent report, Zhang et al show that Th2 cells are more resistant to either TRAIL- or FasL-mediated apoptosis than Th1 cells. Likewise, the Th2 cytokine IL-4 has been shown to protect tumor cells from FasL-mediated apoptosis. Among genes that have been suggested to be modulated by Th2 cytokines is cFLIP, a cytoplasmic protein highly homologous to caspases 8 and 10 that is a potent inhibitor of death-receptor mediated apoptosis. The Th2 cytokine IL-4 has been shown to protect solid tumor cells from FasL-mediated apoptosis via up-regulation of antiapoptotic proteins including FLIP. cFLIP has been shown to be strongly expressed in skin biopsies of MF patients by immunohistochemistry. The same study reported cFLIP expression in the PBL of patients with SzS without specifically looking at CD4+CD26 cells and at levels and frequency not distinct from that found in healthy donor PBL. In the present work, we quantified cFLIP expression in sorted CD4+CD26 tumor cells from the 5 FasL-mediated apoptosis-resistant, Fas-expressing patients with SzS. cFLIP overexpression was found in the 5 tested CD4+CD26 cells from Fas-expressing FasL-resistant patients with SzS, irrespective of the tumor burden. Such an overexpression was not observed in CD4+CD26 cells from FasL-sensitive patients with SzS, healthy donors, and CTCL cell lines, suggesting
that, as reported for other cancers, increased expression of cFLIP may contribute to defective death-receptor signaling. Whether Th2 cytokines such as IL-4 are responsible for the high levels of cFLIP mRNA observed selectively in the CD4+/CD26- cell population of patients with SzS remains to be determined. Nevertheless, the observed increased cFLIP expression in SzS is likely to be of relevance in disease pathogenesis as suggested in mouse models of lymphoma in which enforced overexpression of cFLIP has been shown to favor tumor progression.21,22 In their recent publication, Braun et al23 show an increased expression of cFLIP in CTCL cell lines compared with acute lymphoid leukemia cell lines. We also found such an increased expression in CTCL cell lines and primary tumor cells from patients with SzS. However, unlike the Western blot data of Braun et al,23 we found, using qRT-PCR, significant differences in cFLIP expression between FasL-resistant CD4+ /CD26- cells from patients with SzS and CD4+/CD26- cells from HD, suggesting that this overexpression may directly contribute to Fas resistance despite conserved Fas expression in patients with SzS.

The death ligand TRAIL is considered to be a promising anticancer agent. Agonistic antibodies against DR4,34 or DR5,35 or recombinant soluble TRAIL16-39 are able to induce apoptosis in cancer cells of various origins in vitro and in vivo without affecting normal cells. TRAIL is currently being evaluated clinically in a phase Ib as an anticancer agent in a variety of solid tumors and hematologic malignancies. Gene expression profiling by qRT-PCR showed an overexpression of TRAIL in progressive CTCL,40 but the expression of TRAIL receptors and sensitivity of SzS primary tumor cells to TRAIL has not been reported to date. Here, we provide novel data revealing that circulating CD4+ cells from patients with SzS are completely resistant to soluble TRAIL and such patients harbor a significant, tumor cell–restricted, loss of TRAIL-receptor 2 (DR5). It is noteworthy that DR5 was expressed by CD4+/CD26- nontumor cells from patients with SzS, thus suggesting that the total resistance to TRAIL is most likely a tumor-associated event. Loss or altered expression of DR5 has been reported previously for certain other cancers.10,11 This data suggests that the use of TRAIL as single anticancer agent may be ineffective in SzS.

In the present work, we have investigated 2 approaches to circumvent the observed resistance of PBL from patients with SzS to apoptosis mediated by Fas signaling. As previously shown for acute myeloid leukemia,41 the use of recently developed soluble hexameric form of FasL, namely MFL, allowed us to induce significant apoptotic cell death in all the tested SzS tumor cell samples. The reason of the increased efficiency of MFL compared with other forms of soluble FasL remains unknown. A possible increase of the recruitment of weakly expressed Fas at the surface of tumor cells by MFL due to its hexameric nature (compared with mono or trimers) or to its own conformation (compared with the SuperFasL hexamer used in this study) may be an explanation. It is noteworthy that the low observed IC50 of MFL on SzS PBL in vitro suggests that there may be a therapeutic window of opportunity for the uses of MFL in the treatment of SzS. Indeed, circulating levels of MFL that were 10 to 100 times higher than the concentrations necessary to kill tumor cells in vitro were attained after injection of MFL in mice, in the absence of severe systemic toxicity.42 Accordingly, MFL treatment of mice bearing xenograft tumors of ovarian cancer resulted in a decrease in tumor growth at tolerated doses. On the basis of these and other favorable results, MFL is currently being tested as an anti-tumor agent in phase I clinical trials.

The 2 main objectives of SzS treatment are (1) to favor the elimination of tumor cells and (2) to restore a normal immune response. Multimodality therapy, which is now the standard of care for advanced CTCL, includes the use of agents that induce apoptosis such as PUVA or, more recently, bexarotene, a retinoid X receptor specific agent.43 Combinations of apoptotic agents and agents targeting the activation of immune cells are known to induce higher therapeutic responses than apoptotic agents alone.44 As an example, treatment associating PUVA and IFN-α leads to a higher clinical response in CTCL patients than PUVA alone. Here we investigated the effect of IFN-α and IFN-γ on the sensitivity of patients with SzS PBL to soluble FasL and could demonstrate that pretreatment with either of these cytokines could increase Fas expression on SzS tumor cells and simultaneously increase their sensitivity to soluble FasL.

Taken together, our observations suggest that resistance to apoptosis induced by the TNF ligands FasL and TRAIL occur in a significant subset of patients with SzS. This may contribute to the observed accumulation of CD4+/CD26- clonal T cells in the disease and should be taken into account for the design of therapeutic strategies in SzS.

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Authorship

Contribution: E.C., K.K., S.R., O.G., M.D., and R.S performed the experiments; A.H.R. and L.E.F. designed the research; E.C. and L.E.F. wrote the article.

Conflict-of-interest disclosure: L.E.F. declares a financial interest in Topo-target, a company whose potential product was studied in the present work. The remaining authors declare no competing financial interests.

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References


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