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Crisscross CTL Induction by SYT-SSX Junction Peptide and Its HLA-A*2402 Anchor Substitute

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To investigate the effects of anchor substitutions in SYT-SSX junction peptide, an HLA-A24 anchor residue (position 9) of the SYT-SSX B peptide (GYDQIMPKK) was substituted to more favorable residues according to the HLA-A24-binding motif. Among four substitutes constructed, a substitute with isoleucine (termed K9I peptide) most apparently enhanced the affinity for HLA-A24 molecule. Subsequent in vitro CTL induction analysis using PBMCs of 15 HLA-A24+ synovial sarcoma patients revealed that the original B peptide allowed to induce synovial sarcoma-specific CTLs from 7 patients (47%), whereas such CTLs were inducible from 12 patients (80%) with K9I peptide. Moreover, the extent of cytotoxicity against HLA-A24+ synovial sarcoma cell lines was higher in K9I peptide-induced CTLs than B peptide-induced CTLs. Influence of anchor substitution on peptide/TCR interaction was evaluated by cytototoxicity assays against autologous cells and tetramer analysis. CTLs induced from a synovial sarcoma patient using K9I peptide did not lyse autologous PHA blasts or EBV-infected B cells. In vitro stimulations of PBMCs from 5 HLA-A24+ synovial sarcoma patients with K9I peptide increased the frequency of T cells reacting with both HLA-A24/K9I peptide tetramer and HLA-A24/B peptide tetramer. In contrast, the frequency of T cells reacting with HLA/HIV-derived peptide tetramer remained low. These findings support the validity in design of anchor residue substitution in SYT-SSX fusion gene-derived peptide, and provide a potential clue to the current stagnation in vaccine trials of fusion gene-derived natural junction peptides. The Journal of Immunology, 2004, 173: 1436–1443.

Specific chromosomal translocations characterize many leukemias, lymphomas, and sarcomas (1, 2). The fusion regions of translocation products are strictly limited to the corresponding tumors, thereby serving as attractive targets for tumor-specific therapies, including immunotherapy (3, 4). Immunogenicity of fusion gene-derived natural peptides has been investigated in various tumors, including chronic myelogenous leukemia (5, 6), synovial sarcoma (7, 8), Ewing’s sarcoma (9), and alveolar rhabdomyosarcoma (9). Vaccination trials of natural junction peptides from BCR-ABL (12 patients with chronic myelogenous leukemia) (10), EWS-FLI1 (12 patients with Ewing’s sarcoma) (9), and PAX3-FKHR (4 patients with alveolar rhabdomyosarcoma) (9) demonstrated the safety of the peptides. However, tumor remission was noted only in 1 patient with Ewing’s sarcoma.

To improve immunogenicity of antigenic peptides, an idea has emerged to design peptides in which substitutions of amino acids are artificially introduced in anchor positions. Such peptide engineering, termed altered peptide ligand (11), has successfully improved the binding affinity for HLA and immunogenicity of natural antigenic peptides in several tumor-associated Ags. In contrast, there is a concern regarding conformational changes in MHC/peptide complex groove by anchor residue modifications, which influence peptide/TCR interaction and subsequent immune responses (12–15).

Using synovial sarcoma as a prototype of translocation-associated sarcomas, we have analyzed the immunogenic properties of SYT-SSX gene-derived peptides (7, 16). In the present study, we introduced single amino acid substitutions at an HLA-A24 anchor residue of a natural SYT-SSX junction peptide and evaluated their affinity to HLA-A24 molecule and property to induce CTLs against synovial sarcoma cells and autologous normal cells. In addition, using HLA/peptide tetramer, we assessed the crisscross-reactivity of CTLs induced with the parental SYT-SSX peptide and its anchor substitute.

Materials and Methods

Abs and cell lines

Abs used were: anti-HLA-A24 mAb C7709A2.6 (17), anti-MHC class I mAb W6/32, and anti-MHC class II mAb L243. C7709A2.6 was a gift from P. Coulie (Universite Catholique de Louvain, Brussels, Belgium). W6/32 and L243 were purchased from American Type Culture Collection (ATCC, Manassas, VA).
Synovial sarcoma cell lines (Fuji, HS-SY-II, and SW982), an erythroleukemia cell line (K562), and a T-B lymphoblast hybrid transfected with HLA-A2402 (T2-A*2402) were used. Fuji cells were obtained from T. Nojima (Kanazawa Medical University, Kanazawa, Japan) (18), HS-SY-II cells were from H. Sonobe (Kochi Medical College, Kochi, Japan) (19), and T2-A*2402 cells were from K. Kuzushima (Aichi Cancer Research Institute, Nagoya, Japan) (20, 21). SW982 and K562 cells were purchased from ATCC. Fuji, SW982, K562, and T2-A*2402 were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS, and HS-SY-II was cultured in DMEM (Sigma-Aldrich) containing 10% FCS. G418 (0.8 mg/ml) was continuously added to the culture medium for T2-A*2402 cells.

**Peptides**

A 9-mer peptide (SS393: GYDQIMPKK; in this study renamed as B peptide), spanning the SYT-SSX fusion region, was synthesized previously (7). Single amino acid substitutions were introduced at the position 9, an HLA-A24 anchor residue, changing lysine (K) to leucine (L), isoleucine (I), phenylalanine (F), and tryptophan (W), according to the preferred HLA-A24-binding motif (22, 23). Consequently, four peptides designed as K9L, K9I, K9F, and K9W were synthesized (Fig. 1). Also, three peptides (EBV: TYGPVFLS, HIV: RYLRDQQLL, and vesicular stomatitis virus (VSV): RGYVYQL) were used for reference. The EBV peptide was derived from EBV latent membrane protein 2 (24), the HIV peptide from HIV envelope (25), and the VSV peptide from vesicular stomatitis virus (26). The EBV and HIV peptides have HLA-A24-binding motif. The VSV peptide binds to mouse class I molecule, but lacks HLA-A24-binding motif.

**Peptide-binding assay**

The affinity of peptides for HLA-A24 molecules was evaluated by cell surface class I stabilization assay described by Kuzushima et al. (20). T2-A*2402 cells (2 × 10⁶) were incubated with 200 μl of RPMI 1640 containing 0.1% FCS and peptides at 1, 10, and 100 μM at 26°C for 16 h and 37°C for 3 h. After washing with PBS, the cells were incubated with mAb C7709A2.6 at 4°C for 30 min, and then with FITC-conjugated goat anti-mouse IgG Ab (Cappel, Aurora, OH) at 4°C for 3 h. After washing with PBS, the cells were incubated with mAb C7709A2.6 at 4°C for 30 min, and then with FITC-conjugated goat anti-mouse IgG Ab (Cappel, Aurora, OH) at 4°C for 30 min. After further washing, the cells were suspended with 1 ml of PBS containing 1% formaldehyde and analyzed with FACSscan (BD Biosciences, Mountain View, CA). Affinity of each peptide for HLA-A*2402 molecule was evaluated by percentage of mean fluorescence intensity (%MFI) increase of the HLA-A*2402 molecule in the following calculation. %MFI increase = (MFI with the given peptide – MFI without peptide)/(MFI without peptide) × 100.

**Participants**

The ethical committees of participating institutions approved this study. Peripheral blood samples were collected from patients who had been diagnosed histologically with synovial sarcoma and had given informed consent. Of these, 15 HLA-A24 samples defined by flow cytometry using mAb C7709A2.6 were subjected to the analysis (Table I).

**CTL induction**

PBMCs were isolated from blood samples of HLA-A24 synovial sarcoma patients using Lymphoprep (Nycomed, Oslo, Norway). After a 2-h incubation in AIM-V medium (InVitrogen, Carlsbad, CA) at 37°C, nonadherent cells were separated from PBMCs and divided into CD8+ cells and CD8- cells using an anti-CD8 mAb coupled to magnetic microbeads (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany). CD8+ T cells were cultured in AIM-V with 100 U/ml rIL-2 (a gift from Takeda Pharmaceutical, Osaka, Japan) for 1 wk. CD8+ cells were cultured in AIM-V with PHA (1 μg/ml) (Wako Biochemicals, Osaka, Japan) and IL-2 (100 U/ml) for 3 days, and without PHA for the following 4 days. On days 7, 14, 21, and 28, CD8+ T cells (1 × 10⁶) were repeatedly stimulated with PHA-activated CD8+ PBMCs (5 × 10⁶) in 2 ml of AIM-V, which had been pulsed with the peptides (50 μg/ml) and human β₂-microglobulin (25 μg/ml) for 2 h and irradiated with 100 Gy. The cytotoxic activity of the responder cells was evaluated on day 35.

**Cytotoxicity assay**

The cytotoxic activity of stimulated CD8+ T cells was measured by 6-h ³¹Cr release assay (7). Target cell lines (Fuji, HS-SY-II, SW982, and K562) were labeled with 100 μCi of ³¹Cr for 1 h at 37°C. T2-A*2402 cells were pulsed with 10 μM B peptide for 1.5 h at 26°C and labeled with ³¹Cr. The stimulated CD8+ T cells were mixed with the labeled target cells in the well at a concentration of 2 × 10⁵ cells/well. After a 6-h incubation, the supernatant was collected and counted.

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Table I. *Clinical picture of participants and CTL induction*

<table>
<thead>
<tr>
<th>Participants</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Location of the Tumor</th>
<th>State of Tumor*</th>
<th>Chemotherapy</th>
<th>SYT-SSX Gene</th>
<th>CTL Induction</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B peptide</td>
<td>K9L peptide</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>69</td>
<td>M</td>
<td>Lower leg</td>
<td>P, MT</td>
<td>Not done</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>M</td>
<td>Upper arm</td>
<td>(P)*, MT</td>
<td>Underway</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>M</td>
<td>Lower leg</td>
<td>(P), MT</td>
<td>Underway</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>F</td>
<td>Thigh</td>
<td>(P), MT</td>
<td>Done</td>
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<td>+</td>
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<tr>
<td>5</td>
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<td>F</td>
<td>Thigh</td>
<td>(P), MT</td>
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<td>+</td>
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<tr>
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<td>7</td>
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<td>9</td>
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<td>11</td>
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<td>14</td>
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<td>M</td>
<td>Thigh</td>
<td>(P), MT</td>
<td>Not done</td>
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<td>+</td>
</tr>
<tr>
<td>15</td>
<td>39</td>
<td>F</td>
<td>Neck</td>
<td>(P), MT, LR</td>
<td>Not done</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* P, primary tumor; MT, metastatic tumor; LR, local recurrence.

Parentheses indicate that the tumor had been present previously, but was free at the time blood sample was taken.
incubation period at 37°C, the release of the $^{51}$Cr label was measured by collecting the supernatant, followed by quantification in an automated gamma counter. The percentage of specific cytotoxicity was calculated as the percentage of specific $^{51}$Cr release: $(\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})/(\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})) \times 100$.

**FIGURE 2.** Affinity of agretope-modified SYT-SSX peptides for HLA-A*2402 molecule. Affinity of each peptide for HLA-A*2402 molecule was evaluated by percentage of MFI (%MFI) increase of the HLA-A*2402 molecule on T2-A*2402 cells.

**FIGURE 3.** Cytotoxicity of CTLs against peptide-pulsed T2-A*2402 cells. PBMCs from a synovial sarcoma patient (case 6) were stimulated four times with B peptide (A) and K9I peptide (B), respectively. Cytotoxicity assays were conducted against T2-A*2402 cells that had been pulsed with B peptide. K562 cells that lack class I molecules were used as a control. CTLs induced with K9I peptide showed higher cytotoxicity against B peptide-pulsed T2-A*2402 cells than did those induced with B peptide.
In blocking experiments, 51Cr-labeled target cells were incubated with W6/32, L243, and C7709A2.6 on ice for 30 min, respectively. CTLs were then added and incubated for 6 h at 37°C. Radioactivity of the culture supernatant was measured, as described above.

In experiments to assess autologous cytotoxicity, two types of cells were prepared from PBMC of a patient (case 14). CD8-negative nonadherent PBMCs were cultured in AIM-V medium containing 1μg/ml PHA (Wako Biochemicals) and 100 U/ml IL-2 for 3 days. After washing, the cells were further cultured in AIM-V medium containing IL-2 (100 U/ml) alone for 4 days. The resultant cells were regarded as PHA blasts. Also, B cells isolated from PBMCs were infected with EBV, as previously described (27). These cells were used as autologous targets of CTLs induced from case 14 patients in cytotoxic assays.

Tetramer construction and FACS analysis

Three HLA-A24/peptide tetramers (HLA-A24/B, HLA-A24/K9I, and HLA-A24/HIV) were constructed according to the method described before (7, 16). Flow cytometric analysis was performed for PMBCs of a patient (case 14). CD8-negative nonadherent PMBCs were cultured in AIM-V medium containing 1 μg/ml PHA (Wako Biochemicals) and 100 U/ml IL-2 for 3 days. After washing, the cells were further cultured in AIM-V medium containing IL-2 (100 U/ml) alone for 4 days. The resultant cells were regarded as PHA blasts. Also, B cells isolated from PMBCs were infected with EBV, as previously described (27). These cells were used as autologous targets of CTLs induced from case 14 patients in cytotoxic assays.

Results

Affinity of agretope-modified SYT-SSX peptides for HLA-A24 molecule

To investigate the effects of anchor residue substitutions in SYT-SSX-derived peptides, we modified the position 9 of the natural peptide (B peptide) to leucine (K9L), isoleucine (K9I), phenylalanine (K9F), and tryptophan (K9W), and then evaluated the affinity of these modified peptides to HLA-A24 molecules. In this assay, the affinity of peptides was measured as the increase in MFI of HLA-A*2402 molecules on the surface of T2-A*2402 cells after peptide pulsation. As shown in Fig. 2, pulsation of a positive control EBV peptide increased MFI of HLA-A*2402 molecules in a range from 82 to 137%, depending on the concentration of the peptide applied. In contrast, pulsation of a negative control VSV peptide resulted in <10% increases in MFI. In a comparison between the agretope-modified peptides and the natural B peptide, all four agretope-modified peptides showed higher MFI increases than did the B peptide when pulsed to T2-A*2402 cells. Notably, K9I peptide had the highest affinity to HLA-A24 molecules, with MFI increases of 60% or more.
FIGURE 5. Inhibition of cytotoxicity by MHC class I mAb and anti-HLA-A24 mAb. Fuji cells (A) and HS-SY-II cells (B) were incubated with anti-MHC class I mAb W6/32, anti-HLA-A24 mAb C7709A2.6, and anti-MHC class II mAb L243, respectively. CTLs induced from PBMCs of case 6 patient using K9I peptide were then added, and cytotoxicity was evaluated. Cytotoxicity was clearly inhibited by W6/32 and C7709A2.6, but not by L243.

FIGURE 6. Cytotoxicity of CTLs against autologous cells. PBMCs from a synovial sarcoma patient (case 14) were stimulated four times with K9I peptide. Cytotoxicity assays were conducted against autologous PHA blast and EBV-infected B cells as well as control cell lines. CTLs induced with K9I peptide showed cytotoxicity to Fuji and HS-SY-II cells, but not to SW982 cells, K562 cells, and autologous cells.
Efficacy and specificity of CTL induction with agretope-modified K9I peptide

We subsequently examined the ability of peptides to induce CTLs, focusing on K9I peptide that showed the highest affinity for HLA-A24 among the four agretope-modified peptides. PBMCs from 15 HLA-A24+ synovial sarcoma patients were stimulated in vitro four times using parental B peptide and K9I peptide, respectively. As depicted in Table I, peptide as well as synovial sarcoma-specific CTLs were successfully induced from 12 patients (80%) with K9I peptide, except 3 patients who have no metastasis or local recurrence. In contrast, such CTLs were inducible from 7 patients (47%) with B peptide. Furthermore, the extent of cytotoxicity was higher in K9I peptide-induced CTLs than B peptide-induced CTLs. Representative results were shown in Figs. 3 and 4.

In blocking experiments using CTLs from case 6 patient, anti-MHC class I mAb W6/32 and anti-HLA-A24 mAb C7709A2.6 apparently inhibited the cytotoxic activity against Fuji cells (Fig. 5A) as well as HS-SY-II cells (Fig. 5B), whereas anti-MHC class II mAb L243 failed to show such inhibitory effects.

FIGURE 7. Crisscross-reactivity of CTLs analyzed by HLA-A24/peptide tetramer. PBMCs of case 14 patient were stimulated four times with B and K9I peptide, respectively. Their reactivity with HLA-A24/B and HLA-A24/K9I tetramers was then analyzed by FACScan.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Naive T Cell HLA-A24/B Tetramer</th>
<th>HLA-A24/B tetramer</th>
<th>HLA-A24/K9I tetramer</th>
<th>HLA-A24/HIV tetramer</th>
</tr>
</thead>
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<tr>
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<td>0.5</td>
<td>0.07</td>
<td>0</td>
</tr>
<tr>
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<td>0.6</td>
<td>0.75</td>
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<tr>
<td>12</td>
<td>0.15</td>
<td>1.3</td>
<td>1.55</td>
<td>0.02</td>
</tr>
<tr>
<td>13</td>
<td>0.21</td>
<td>0.46</td>
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<tr>
<td>14</td>
<td>0.47</td>
<td>0.6</td>
<td>0.62</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table II. Cross-reactivity of K9I-induced CTLs
To determine possible cross-reactivity of K9I peptide-induced CTLs to normal cells, cytotoxicity was examined on autologous PHA blast and EBV-infected B cells. As shown in Fig. 6, K9I peptide-induced CTLs exhibited substantial cytotoxicity to Fuji and HS-SY-II cells, but not to SW982 cells, K562 cells, and autologous cells.

Crisscross-reactivity of CTLs induced with B and K9I peptides

Finally, we analyzed crisscross-reactivity of CTLs induced with B and K9I peptides using PBMCs from five patients with synovial sarcoma. Table II summarizes the results of the HLA-A24/peptide tetramer analysis. As shown, in vitro stimulations with K9I peptide increased the frequency of T cells reacting with both HLA-A24/K9I peptide tetramer and HLA-A24/B peptide tetramer, except case 10 patient. In contrast, the frequency of T cells reacting with HLA/HLA peptide remained low. Conversely, as shown in Fig. 7, in vitro stimulations with B peptide resulted in increases in frequency of T cells reacting with both HLA-A24/K9I peptide tetramer and HLA-A24/B peptide tetramer.

Discussion

In the present study, we substituted an HLA-A24 anchor residue (position 9) in the SYT-SSX natural junction peptide, B peptide, to demonstrate that substitution of lysine to isoleucine: 1) enhances the affinity for HLA-A24 molecule; 2) improves the capacity of the peptide to induce synovial sarcoma-specific CTLs; and 3) retained a crisscross-reactivity of CTLs with original SYT-SSX-sequence. These findings support the validity in design of anchor residue substitution in SYT-SSX fusion gene-derived peptide, and provide a potential clue to the current stagnation in vaccination trials of fusion gene-derived natural junction peptides.

Improved immunogenicity of antigenic peptides by means of anchor residue substitutions has been reported on various tumor-associated Ags, including gp100 (28–30), Melan-A/MART-1 (31–33), HER2/neu (34), NY-ESO-1 (35, 36), MUT-1 (37), and mutant ras p21 (38). This approach is based on the assumption that each residue in the peptide binds independently to class I MHC and TCR, and thus substitution of anchor residues increases the binding affinity without interfering with peptide recognition. In contrast, recent studies using recombinant Abs (12), T cell clones (13), and crystallographics (14, 15) have revealed conformational changes in MHC/peptide complex groove by anchor residue modifications, which influence peptide/TCR interaction. In this regard, we assessed the crisscross-reactivity of K9I and B peptide using HLA-A24/peptide tetramer, the structural mimic of HLA/peptide complex on the cell surface. Obviously, four-time stimulations of T cells with K9I or B peptide concurrently increased the frequency of T cells reacting with both K9I and B peptide. It should also be noted that vaccinations of B peptide (1 mg/ml) in our ongoing phase I trial resulted in increases in the frequency of T cells in both HLA-A24/B peptide and HLA-A24/K9I peptide tetramers (K. Ida and S. Kawaguchi, unpublished observation), further supporting the crisscross-reactivity of these two peptides. CTLs induced with K9I did not show cytotoxicity against autologous normal cells, diminishing the likelihood of the possible autoimmunity by amino acid substitution.

In our previous study (7), reactivity of HLA-A24/B peptide tetramer to circulating T lymphocytes in synovial sarcoma patients was significantly associated with the present and past history of distant metastasis. In the present analysis, CTLs were also inducible from all seven patients who have history of distant metastasis using K9I peptide. In contrast, CTLs were induced from four of seven patients who have no tumor relapse. These findings support our previous assumption that systemic blood-borne metastasis is an important event for SYT-SSX gene products to prime SYT-SSX-specific CTLs.

It should be noted that CTL responses were inducible with B peptide from 47% of patients, even though this peptide binds poorly to HLA-A24 molecules. In this regard, the extent of positive CTL responses induced with B peptide was not very high, as can be seen in Figs. 3A and 4A, as well as in our previous study (7). Such relatively low responses, although determined as positive in 47% of patients, may reflect low affinity of B peptide as a limitation of the reverse immunology approach.

Because of the limited availability of PBMCs, other variant peptides (K9L, K9F, and K9W) were not tested for CTL induction. Also, failure in obtaining CTL clones by stimulation with B or K9I peptide hampered us to evaluate the affinity of the TCR on two different CTLs to the peptides. These analyses would be helpful in delineating the mechanism of enhanced T cell stimulation by anchor substitution.

In conclusion, the present study evaluated the cytotoxicity and crisscross-reactivity of CTLs induced with SYT-SSX-derived junction peptide and its anchor substitute. Our results extended the previous concept of anchor substitution toward fusion gene-derived peptides in synovial sarcoma. Because the present study serves as a preliminary report, it is essential to determine the efficacy of anchor substitution in vivo by clinical studies.

Acknowledgments

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References


