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The Adjacent Flanking Region Plays a Critical Role in Facilitating the Presentation of the *Listeria monocytogenes* Product lemA to H2 M3wt-Restricted, Peptide-Specific Murine CD8 Cells

Roger J. Kurlander,* Elizabeth Chao,* Janet Fields,* and Chandrasekaran Nataraj†

Mice infected with *Listeria monocytogenes* (LM) generate CD8 effectors specific for f-MIGWII, the amino terminus of the bacterial product lemA presented by the class Ib MHC molecule H2 M3wt. lemA has several distinctive properties: 1) it is readily presented as an exogenous Ag in the absence of bacterial infection; 2) it is processed by a TAP-independent pathway, which is sensitive to chloroquine, pepstatin, and brefeldin; and 3) the immunogenic portion of the molecule is extremely resistant to proteolytic degradation even by proteinase K. To assess the structural basis for these findings, we expressed a truncated variant (t-lemA) containing the amino-terminal hexapeptide and the subsequent 27 amino acids linked to a histidine tail in *Escherichia coli*, purified the product by affinity chromatography. Purified t-lemA could be presented to f-MIGWII-specific effectors by macrophages and fibroblasts at 1–10 nM. Unlike f-MIGWII, which binds directly to H2 M3wt, t-lemA required processing by a chloroquine-, pepstatin-, and brefeldin-sensitive pathway. Brefeldin sensitivity often implies endogenous processing in the cytoplasm, but several lines of evidence suggest translocation to the cytoplasm and proteosomal degradation are not critical for t-lemA presentation. Unlike f-MIGWII, t-lemA was profoundly resistant to proteinase K, and, using 35S-labeled t-lemA, we could identify the region from position 1 to ~30 as the protease-resistant element. Thus, the hydrophobic peptide sequence following f-MIGWII can account for the unusual properties of lemA noted above. Analogous modification could be used to alter the properties of other peptide Ags presented by class I MHC products. The *Journal of Immunology*, 1999, 163: 6741–6747.
the mechanisms underlying these phenomena merit further evaluation. Since previous studies have all been performed using whole bacteria or crude bacterial extracts containing a variety of potentially bioactive contaminants, we have cloned a truncated variant (t-lemA) containing only the first 33 amino acids of lemA. These code the immunogenic N-terminal f-MIGWII hexapeptide, and the subsequent 27 amino acids, which are essential for preservation of the initial formyl group during biosynthesis (18), and for maintaining the hydrophobic character of the Ag. This cassette was inserted in front of a carboxy-terminal histidine tag in the expression vector pET24a to facilitate purification and expression in Escherichia coli. The following studies demonstrate that the resulting recombinant 5.4-kDa Ag, purified to near homogeneity by metal chelation chromatography, recreates the biochemical and functional properties of crude lemA in the absence of other bacterial proteins or lipids. Thus, the unusual properties of lemA can be fully accounted for by the covalent association of the immunogenic f-MIGWII sequence with the hydrophobic, protease resistant scaffolding provided by the lemA1-33 sequence. Manipulation of this simple motif may prove to be a useful strategy for altering the biochemical stability and processing requirements of other class I-restricted peptides.

Materials and Methods

Chemical reagents

PK, brefeldin, chloroquine, phosphomolybdic acid, phosphatidyl ethanolamine, cardiolipin, phospholipid standards, OVA, polyethylene glycol 1000, and PMSF were purchased from Sigma (St. Louis, MO). Pepstatin, isopropyl β-D-thiogalactoside (IPTG), N-α-cytoleucine (OG), restriction enzymes NdeI and SalI, and kanamycin were purchased from Boehringer Mannheim (Indianapolis, IN). Silica HPTLC plates were produced by EM Science (Gibbstown, NJ). Synthetic f-MIGWII was prepared by Peptide Technology (Gaithersburg, MD).

Animals

Seven- to 12-wk-old male C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a barrier facility within the National Institutes of Health. TAP-deficient B6 mice (19) originally obtained from L. Van Kaer were kindly provided by J. Bennink (Bethesda, MD). All animal strains and cell lines, unless specifically indicated, expressed H2 M3*.

Bacteria

LM of strain 10403-S (20) was used to prepare DNA template and heat-killed LM (11). E. coli strains INVoFA (Invitrogen, San Diego, CA) and BL21(DE3) (Novagen, Milwaukee, WI) were used in the amplification and expression of plasmids containing t-lemA.

Preparation of t-lemA

The lemA sequence from nucleotides 28–383 (5) was cloned from LM DNA by PCR using the synthetic oligonucleotide primers 5'-ATTACATA TGATAAGATGTTATGGCTCGT-3' and 5'-GGCCAGAC CGTATCCGTATTTTACGCTG-3'. To this end, a small amount of bacterial DNA (from microwave-disrupted bacteria) and primers (0.2 μM final concentration) was subjected to 30 cycles of PCR using preformulated beads containing Taq polymerase, nucleotides, and buffer (Pharmacia Bio- tech, Piscataway, NJ). The resulting 108-nt insert was excised from the plasmid using NdeI and SalI; gel was purified in a 3% agarose gel and inserted into the expression vector pET24a (Novagen). The resulting recombinant t-lemA-pET24a plasmid was expanded in E. coli strain INVoFA, purified, and then transfected into BL21(DE3).

Expression of t-lemA protein

The final product, t-lemA, is 46 amino acids in length with a predicted molecular mass of 5.4 kDa and the sequence MIGWIIAAAYVVLVLIV YGLNLSVVKYRNVSKDLKAALHEHHHHH. To express this product, BL21(DE3) transfected with t-lemA-pET 24a was incubated with Luria-Bertani (LB) medium containing 1 mM IPTG for 3 h using conditions suggested by the manufacturer (Novagen). Treated bacteria were centrifuged at 18,000 × g for 15 min, washed in 20 mM Tris (pH 8.0), lysozyme treated (0.1 mg/ml for 1 h), and disrupted by sonication. The particulate fraction and supernatant were separated by centrifugation (39,000 × g for 20 min), and the former was solubilized in 4% OG/20 mM Tris (pH 8)/100 mM NaCl. After centrifugation to remove residual particulates, the solubilized product was diluted 4-fold in 20 mM Tris (pH 8), bound to Talon Metal Affinity Resin (Clontech Laboratories, Palo Alto, CA), washed with 1% OG/20 mM Tris/25 mM NaCl, and finally eluted using 1% OG/20 mM Tris/100 mM imidazole (pH 8). Ag-rich fractions identified by SDS-PAGE, were pooled, dialyzed extensively in water to remove detergent, and equilibrated in PBS for bioassay or with 20 mM Tris (pH 8) for protease treatment. Despite its low m.w., t-lemA could be fully recovered after dialysis, presumably because of self-aggregation of the hydrophobic molecule in an aqueous environment.

We used 16.5% polyacrylamide gels equilibrated in SDS/Tris/tricine buffer with polyamide m.w. standards (Bio-Rad, Hercules, CA) and Geleco Blue stain (Pierce, Rockford, IL) to estimate t-lemA size and monitor its purity. Protein concentrations were measured after Ag solubilization in SDS using the micro-bicinchoninic acid (BCA) assay reagent (Pierce). To screen for the presence of lipids, varying amounts of Ag were spotted on silica gel TLC plates along with defined amounts of phosphatidyl ethanolamine, cardiolipin, and a mixture of phospholipid standards. Ag and standards were chromatographed using a moving phase mixture of chloroform, methanol, water, and acetic acid (25:15:4:2). After drying, the plates were sprayed with phosphomolybdic acid and heated to 110°C to visualize lipid/phospholipid bands.

For selected studies, t-lemA-transfected bacteria were grown in LB medium supplemented with 5 μg/ml [35S]methionine (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting labeled t-lemA product was purified as described above, and then subjected to bioassay and protease treatment in the usual manner.

Cell culture

All cell lines were maintained in RPMI 1640 supplemented with 10% FCS, 1 mM L-glutamine, 200 U/ml penicillin, 200 μg/ml streptomycin, and 50 μg/ml 2-ME (R10). The t-lemA-specific, H2 M3*-restricted CD8 cell line CN.8 was maintained by weekly restimulation with heat-killed LM, irradiated splenocytes, and recombinant IL-2 as described previously (7, 17). To prepare macrophages, bone marrow from the femurs of B6 or TAP-deficient mice was incubated in vitro for at least 5–7 days in 100-mm petri dishes containing R10 supplemented with 100 μM L929-conditioned medium (21). Macrophages harvested from these plates by gentle washing were plated in 96-well flat-bottom microtiter plates at 3.5 × 104/well for use in bioassays.

Fibroblast cell line L929 was obtained from American Type Culture Collection (Rockville, MD). LK6/L929 cells transfected with H2K2* (22), and B3Z, an OVA-specific T cell hybridoma, which produces β-galactosidase in response to the peptide SIINFEKL presented by H2K b (23), were provided by J. Yewdell and J. Bennink (National Institute of Allergy and Infectious Disease, Bethesda, MD). B10.CAS2 (H2 M3*–/–) and CM3, a B10.CAS2 fibroblast line transfected with H2 M3* (24, 25), were provided by K. Fischer Lindahl (University of Texas Southwestern Medical Center, Dallas, TX). 13S2, a B10.CAS2 cell line transfected with a chimeric class I HMC molecule coding the first and second domains of H2 M3* and the third domain derived from Ld (26), was provided by S. Shawar and R. Rich (Baylor Medical Center, Houston, TX).

Osmotic loading

A hypersomolar buffer containing RPMI 1640 medium supplemented with 10% polyethylene glycol 1000, 0.5 M sucrose, and 10 mM HEPES (pH 7.2) was prepared as described previously (27). LK6 cells were incubated in this buffer supplemented with OVA or t-lemA for 10 min at 37°C. Cells were then washed twice with complete medium and incubated for 6 h at 37°C. Control cells were incubated with Ag in isosmotic buffer. Ag presentation by treated cells was then assayed using the standard methods described below.

In the original description of osmotic loading of Ags, Moore et al. (27) exposed loaded cells to hypotonic media for 5 min to enhance osmotic disruption. In our preliminary studies using OVA presentation as a model, we found this step markedly decreased LK6 viability without increasing Ag presentation. Therefore, it was deleted from our loading protocol.

Quantitation of t-lemA presentation to CN.8

Macrophage or fibroblast monolayers (3.5 × 104/well in flat-bottom 96-well plates) were preincubated with varying quantities of purified t-lemA...
or medium alone for 6 h at 37°C. CN.8 cells (1 × 10⁷/well) were then added, and lysis was quantitated 6 h later using the neutral red uptake method (11). The results were expressed in percent lysis of macrophages or fibroblast monolayers. All findings presented were confirmed in at least three independent experiments.

In studies assessing the impact of metabolic inhibitors, target monolayers were pretreated with each inhibitor for 30 min before the addition of Ag. Six hours later, inhibitors and Ag were washed away, and lysis by CN.8 was quantitated as described above in the presence of brefeldin (0.5 µg/ml) to prevent “breakthrough” processing after removal of the inhibitors during the lysis phase of the assay.

**Quantitation of OVA presentation to B3Z**

Varying numbers of LKb pretreated with OVA and/or hypertonic medium as described above were incubated with 1 × 10⁵ B3Z cells overnight in microtiter wells. β-Galactosidase production by B3Z was then measured spectrophotometrically at 570 nm after the addition of 0.15 mM chloromethyl ketone (CMK) and p-nitrophenol red β-d-galacto- pyranoside in PBS/0.5% Nonident P-40 (23).

**PK treatment of f-MIGWII and t-lemA**

To assess protease sensitivity, f-MIGWII and t-lemA, adjusted to 20 µg/ml, were incubated at defined substrate to protease ratios (w/w) with PK in a buffer containing 20 mM Tris (pH 8) and 1 mM CaCl₂ for 18 h at 37°C (17). The protease-treated and control f-MIGWII preparations were biosayed directly at 1500–50,000 dilutions. Because t-lemA preparations could not be diluted as extensively for bioassay, they were treated with 1 mM PMSF to inactivate PK and then dialyzed to remove Tris and PMSF before bioassay. In studies addressing the impact of PK on construct size, [35S]methionine-labeled t-lemA preparations were incubated with varying amounts of PK, and the resulting mixture was analyzed by SDS using Gelcode blue staining to define the pattern of protein fragmentation. A phosphor imager (Fuji Imaging, Tokyo, Japan) was used to assess the size and quantity of [35S]labeled fragments on the same gel.

**Results**

**Preparation of t-lemA**

After IPTG treatment, transformed BL21(DE3) bacteria produced a recombinant product, which could be detected in bacterial lysates using SDS-PAGE (Fig. 1). Detectable amounts of this band were not present in culture supernatants (data not shown). After bacterial sonication, t-lemA remained with the particulate fraction, but it could be solubilized using OG and purified to near homogeneity by metal chelation-affinity chromatography (Fig. 1, lane C) with a yield of 2–3 mg/L of bacterial culture. After dialysis to remove detergent, purified t-lemA formed a microparticulate suspension in water. Using TLC with phosphomolybdic acid staining, we could detect no residual phospholipid contamination of this product with a sensitivity of <0.1 µg phospholipid/µg t-lemA.

**t-lemA can be presented to CN.8 by macrophages or fibroblasts**

CN.8 lysed t-lemA-treated B6 macrophages and L929 fibroblasts in a dose-dependent manner. Half-maximal lysis of the former required 10 nM t-lemA; ~10-fold more Ag was required to produce comparable lysis of fibroblasts. The immunogenic peptide f-MIGWII, by comparison, produced lysis at considerably lower concentrations (Fig. 2). CN.8 also lysed the H2 M3wt-positive fibroblast line CM3, but not B10.CAS2 fibroblasts expressing only H2 M3αα (data not shown), confirming that Ag recognition is H2 M3wt dependent.

**Metabolic requirements for presentation of t-lemA**

We have shown previously that native lemA presentation by macrophages is inhibited by chloroquine, ammonium chloride, pepstatin, and brefeldin (11). Processing of t-lemA by macrophages and fibroblasts was also inhibited by each of these agents (Fig. 3, A and C). Because f-MIGWII presentation is unaffected by chloroquine, ammonium chloride, pepstatin, and only modestly inhibited by brefeldin (Fig. 3, B and D), t-lemA bioactivity cannot be attributed to inadvertent contamination with this entity. To assess the possible influence of the α3 domain of H2 M3wt in shaping t-lemA processing, we tested the function of 13S2, a B10.CAS2 fibroblast cell line transfected with a chimeric H2 M3wt molecule bearing α1 and α2 domains from H2 M3αα and an α3 domain derived from H2Ld. Like cells expressing the native H2 M3wt molecule, 13S2 presented exogenous t-lemA (but not f-MIGWII) in a pepstatin-sensitive manner (Fig. 3, E and F).

Using brefeldin to halt processing at varying time points, we could demonstrate that t-lemA processing by macrophages requires 2–4 h (Fig. 4).

**Role of cytoplasmic translocation and TAP in t-lemA processing**

The H2Kβ-restricted presentation of exogenous OVA by nonprofessional APC is critically limited by the failure of this product to gain access to the cytoplasm. Osmotic loading, which facilitates the transfer of ingested proteins into the cytoplasm, dramatically increases OVA presentation in this setting (28). As expected, this maneuver
enhanced OVA presentation by LK fibroblasts (Fig. 5A). The same procedure however, did not affect lemA presentation (Fig. 5B).

OVA processing after osmotic loading was blocked by lactacystin (29), an inhibitor of proteosomal proteolysis (data not shown). By contrast, t-lemA processing was lactacystin-resistant (Fig. 6A). TAP-deficient macrophages were as proficient as wild type in presenting t-lemA to CN.8 (Fig. 6B).

**PK-mediated fragmentation of t-lemA**

Although the pepstatin sensitivity of processing observed in Fig. 3 implies t-lemA must undergo proteolytic cleavage within APC, like native lemA (17), it is very resistant to proteolytic degradation in vitro. Indeed, bioactivity was enhanced by PK treatment, with or without 1% SDS (Fig. 7B). This protease resistance is not intrinsic to the f-MIGWII sequence itself, since its bioactivity is totally lost after incubation at comparable protein to enzyme ratios (Fig. 7A).

Although PK did not diminish t-lemA bioactivity, it clearly did trim t-lemA from its initial molecular mass of 5.4 kDa to from ~3.5 to 4.5 kDa (Fig. 8, upper panel). Because the only methionine in t-lemA is located at its amino terminus, we used [35S]methionine-labeled lemA and a phosphor imager to assess the susceptibility of the immunogenic amino-terminal portion of the molecule to proteolytic cleavage. In repeated studies, the radioactivity associated with intact t-lemA (Fig. 8, lower panel, lane 1) could be completely accounted for by the 3.5- to 4.5-kDa fragments generated by PK treatment (Fig. 8, lower panel, lanes 2–5). Because proteolytic cuts close to the amino terminus would generate much smaller radioactive fragments with a net loss of radioactivity from the residual 3.5- to 4.5-kDa bands, PK-mediated cleavage must be confined almost exclusively to the carboxyl-terminal portion of the molecule. Although PK substantially reduced Ag size, presentation of the resulting fragments could be potently blocked by pepstatin and brefeldin (Fig. 9), indicating further processing was still required for Ag presentation.

**Discussion**

Many instances of class I MHC product-dependent presentation of exogenous Ags (including other *Listeria* proteins) can be explained by the shunting of Ag from endosomal vesicles into the
cytoplasm where peptide generation and transport to the endoplasmic reticulum are mediated via the endogenous pathway (12, 30). lemA is hydrophobic and particulate, and both of these properties can facilitate promiscuous shunting from early endocytic vesicles to the cytoplasm (31–34). Yet lemA presentation does not appear to be mediated in this manner. Spontaneous shunting (in the absence of invasive infection) has been noted predominantly in professional APC such as macrophages and dendritic cells (30, 35, 36), but in our studies fibroblasts also processed lemA quite readily. Presentation by this mechanism is often lactacystin dependent and almost always TAP dependent, but lemA presentation is neither. Finally, direct translocation of unprocessed t-lemA into the cytoplasm by osmotic loading does not enhance presentation. This suggests that the cytoplasmic machinery may be unable to convert this particular Ag into an immunogenic peptide. Although the current studies do not rule out Ag entry into the cytoplasm at some point in processing, the observed pattern is more consistent with t-lemA degradation somewhere within the endosomal-lysosomal compartment.

In the native lemA molecule, the localization of the f-MIGWII sequence immediately in front of a hydrophobic sequence (from positions 7 to 26) and several cationic amino acids (at position 27, 29, and 31) strongly influences the biosynthesis and localization of the final product. Lacking a leader sequence or a consensus signal peptidase cleavage site, the amino-terminal portion of the molecule presumably remains intact after biosynthesis (5), and based on precedent from other bacterial proteins, probably forms a membrane-spanning domain with an Nout-C in topology (37). By physically transporting the amino terminus away from cytoplasmic deformylases and proteases, localization of the immunogenic N terminus on the outer side of the bacterial membrane may be critical in preserving the N-formyl group characteristic of lemA (18). More relevant to the issue of processing, Lenz and Bevan (5) have speculated that the exogenous presentation of lemA might be critically linked to the vulnerability of the exposed amino terminus of ingested bacteria to release by endosomal proteases. Untethered from the bacterial surface, the resulting fragments might then bind to H2 M3β in a TAP-independent manner within the endosomal compartment.
Although this proposal is a priori plausible, the current studies demonstrate that processing is not linked to any specific orientation of lemA within the bacterial membrane. Recombinant t-lemA retained the identical pattern of processing (chloroquine, ammonium chloride, pepstatin, and brefeldin sensitivity, and TAP independence) noted in earlier studies using crude bacterial preparations (5, 11), despite disruption of native spatial orientation and separation of the purified molecule from other bacterial components. More important, presentation cannot be explained simply by facile cleavage of f-MIGWII from lemA. To the contrary, in the context of the t-lemA molecule, the proteolytic release of an immunogenic fragment is not easily achieved (Fig. 9).

We speculated previously, based on studies using a relatively crude LM extract, that this protease resistance might reflect steric protection of the molecule by closely associated phospholipid (7). This explanation now seems unlikely since affinity-purified t-lemA is free of stoichiometrically significant quantities of these substances. Protease resistance is not simply a consequence of poor solubility, since Ag to stoichiometrically significant quantities of these substances. Protease resistance is not simply a consequence of poor solubility, since Ag remained resistant to degradation even after solubilization in 1% SDS, a maneuver that increases the proteolytic sensitivity of many peptide products. Because the smallest fragments generated by PK are about 30 amino acids in length, we suggest this protease resistance reflects the hydrophobicity of the first 25 amino acids. Presumably, hydrophobic interactions within this core region impede initiation of proteolysis at the amino terminus or further stepwise cleavage from the other end. It is impressive that despite this protease resistance in vitro, t-lemA can be effectively degraded into immunogenic fragments within APC. Because this processing can be inhibited by pepstatin, it presumably is mediated at least in part by cathepsin D, an enzyme also linked to the processing of peptides for presentation to class II MHC products (38).

Numerous previous studies, recently reviewed by Jondal et al. (12), have documented TAP-independent, class I MHC product-restricted presentation of exogenous Ags. This phenomenon has been observed in studies of Ags associated with intact bacteria or latex beads (39); with viruses including hepatitis B virus (40), Sendai virus (41), lymphocytic choriomeningitis virus, and vesicular stomatitis virus (42); and with peptides bound to heat shock proteins (43). In some cases, presentation is only observed using professional APC, but nonprofessional APC such as T2 and RMA-s cells also possess the machinery for processing some of these Ags. Although the sensitivity of these models to lysosomotropic amines and/or inhibitors of endosomal protease activity is variable, presentation has typically been brefeldin independent, implying peptide binding to a pre-existent, recycling pool of Ag-presenting molecules. Indeed, in several of these models, there is strong evidence for binding to class I MHC molecules recycling from the cell surface (39, 44) or even direct binding of regurgitated peptide molecules to surface MHC molecules (34, 45). Although the pool of recycling molecules can be modestly reduced by brefeldin treatment (39), the particular sensitivity of lemA processing to low doses of brefeldin noted here distinguishes it from prior examples of extracytoplasmic processing and presentation.

Because little is known about how newly synthesized H2 M3wt moves within the cell after export from the Golgi apparatus, the brefeldin sensitivity of t-lemA presentation could reflect specialized trafficking of this MHC product to an endosomal site adapted for peptide loading; however, this is unlikely. Directed intracellular trafficking of CD1, a β-2 microglobulin-linked molecule known to undergo peptide loading in the endosomal compartment, is critically linked with the presence of a YYXZ peptide motif in its cytosplasmic tail (46). H2 M3wt has a truncated (8-amino acid) cytosplasmic tail lacking such a sequence (4, 47). Furthermore, the current studies demonstrate that chimeric molecules expressing the α3 domain of H2Lδ (a classical molecule which is not known to traffic extensively in the endosomal compartment) retain the capacity to present lemA in a similar manner.

The brefeldin sensitivity of lemA presentation could also reflect distinctive trafficking of t-lemA itself. In light of its unusual protease resistance, the generation of immunogenic peptides may not be completed until the Ag has entered into a relatively late endosomal site. If recycling surface MHC molecules do not readily enter this compartment, Ag reaching this “point of no return” might only be presented if it gains access to a newly exported (brefeldin-sensitive) pool of H2 M3wt within the endosomal vesicle itself or some other internal compartment. Alternatively, brefeldin might interfere with presentation by inhibiting the transport of H2 M3wt-f-MIGWII complexes formed deep within the cell back to the cell surface (48). Although the brefeldin sensitivity strongly suggests peptide is not being bound to a rapidly recycling population of surface-derived H2 M3wt molecules, additional studies will be needed to define how and where t-lemA-derived immunogenic fragments gain access to H2 M3wt within the cell.
Independent of its significance in shaping intracellular processing within APCs, the proteasome resistance of t-lemA may significantly affect its pharmacologic stability in vivo. Short peptide Ags are highly bioactive, but potentially very vulnerable to degradation by environmental proteases. On the other hand, intact proteins often are more protease resistant, but may be processed very inefficiently as exogenous Ags. Hydophagic particulate Ags like t-lemA could be a relatively stable but potent immunogens in vivo. In future studies, it will be of interest to assess whether t-lemA can also stimulate the generation of immune effectors in vivo and whether the scaffolding provided by the lemA sequence from 7 to 33 can favorably alter the presentation and processing sensitivity of other immunogenic peptides. If so, it may be possible to rationally exploit the properties of lemA in other settings. Chimeric products might be useful Ags in inducing CD8 responses in sites such as mucosal surfaces where there may be high proteolytic activity.

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References


