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Three mitogen-activated protein kinase pathways are up-regulated during the activation of T lymphocytes, the extracellular signal-regulated kinase (ERK), Jun NH₂-terminal kinase, and p38 mitogen-activated protein kinase pathways. To examine the effects of blocking the ERK pathway on T cell activation, we used the inhibitor U0126, which has been shown to specifically block mitogen-activated protein kinase/ERK kinase (MEK), the kinase upstream of ERK. This compound inhibited T cell proliferation in response to antigenic stimulation or cross-linked anti-CD3 plus anti-CD28 Abs, but had no effect on IL-2-induced proliferation. The block in T cell proliferation was mediated by down-regulating IL-2 mRNA levels. Blocking Ag-induced proliferation by inhibiting MEK did not induce anergy, unlike treatments that block entry into the cell cycle following antigenic stimulation. Surprisingly, induction of anergy in T cells exposed to TCR cross-linking in the absence of costimulation was also not affected by blocking MEK, unlike cyclosporin A treatment that blocks anergy induction. These results suggest that inhibition of MEK prevents T cell proliferation in the short term, but does not cause any long-term effects on either T cell activation or induction of anergy. These findings may help determine the viability of using mitogen-activated protein kinase inhibitors as immune suppressants.

T

Since Elk-1 has been implicated in the up-regulation of the c-fos gene, and the transcription factor ATF-2 can form heterodimers with Jun subunits, this suggests some involvement of the p38 MAP kinase pathway as well in AP-1 binding and IL-2 synthesis.

The role of the MAP kinase pathways in inducing T cell tolerance is also of interest. Th1 cells can be rendered unresponsive (anergic) by TCR occupancy in the absence of costimulation. This results in suboptimal activation of the T cells, with no IL-2 production and no resultant proliferation (22). In addition, such cells are unresponsive to subsequent stimulation with normal APC plus Ag (22). In response to Ag plus APC, anergized T cells have been shown to undergo early events characteristic of T cell activation (23, 24), to have normal surface expression of TCR, CD4 (24), and CD28 (D. R. DeSilva and Marc K. Jenkins, unpublished data), but to fail to accumulate IL-2 mRNA.

Of the three MAP kinase pathways described in mammalian cells, the ERK pathway is the best defined. This pathway is up-regulated via Ras, which is transiently activated in response to TCR binding. Upon activation, Ras complexes with, and in turn activates the serine-threonine kinase Raf-1, which then phosphorylates the MAP kinase kinase, MEK (25). MEK, a dual-specific kinase, activates ERK by phosphorylating it on two critical residues, Tyr183 and Thr185. ERK then translocates into the nucleus and regulates the activities of several nuclear transcription factors.

In this study, we examined the effects of blocking the ERK pathway on T cell activation and anergy by using an inhibitor, U0126, which specifically inhibits the ability of MEK to phosphorylate ERK without affecting the JNK and p38 pathways. This compound blocked T cell proliferation in response to antigenic stimulation by decreasing IL-2 mRNA levels, but did not block the response of cells to exogenously added IL-2. Blocking the ERK pathway during T cell activation did not affect the response of the T cells to subsequent restimulation. Induction of anergy was also not affected by MEK inhibition, suggesting that immune suppressants that target the ERK pathway would not cause long-term perturbation of the immune response.
Materials and Methods

**Mice and reagents**

B10.BR/SgSn mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c mice were purchased from Charles River Laboratories (Raleigh, NC). Single-cell suspensions of whole spleen obtained from these mice were used as APCs for maintenance of Th1 clones and in proliferation assays. The compounds, U0126, U0124, PD098059, and SK&F8602, were synthesized by DuPont Merck Chemistry Department (Wilmington, DE).

**T cell clones**

**A.E7.** This is a B10.A-derived CD4^{+}8^{-}, CD28^{-} murine Th1 clone (26) that expresses a V_{beta}Val11 TCR. A.E7 cells produce IL-2, IL-3, and IFN-\gamma upon stimulation with the C-terminal cyogen bromide fragment (residues 81–104) of pigeon cytochrome c or a synthetic peptide based on the moth cytochrome c sequence called DASP (27) bound to I-E^{k} molecules on the surface of APC. The DASP peptide was synthesized using the Merrifield method at the University of Minnesota (Minneapolis, MN) microchemical facility.

**Th17.** This murine Th1 clone was derived from BALB/c mice immunized s.c. with the influenza virus A/Puerto Rico/8/34 (PR8), as described (28). It is CD4^{+}8^{-}, CD28^{-}, and produces IL-2 and IFN-\gamma upon stimulation with the hemagglutinin molecule of influenza virus PR8. The viral Ag was in the form of allantoic fluid from virus-infected embryonated hen eggs. Virus titers were determined by agglutination of chicken erythrocytes and are expressed as hemagglutinating units (28).

Both A.E7 and Th17 cells were maintained in 24-well plates on a “rest-stimulation” protocol (26) using mitomycin C-treated APC, Ag, and human rIL-2 (Boehringer Mannheim, Indianapolis, IN). Cells used in experiments were rested at least 5 days after addition of IL-2 and were separated from debris and dead APC by Histopaque 1077 (Sigma, St. Louis, MO) density gradient centrifugation.

**Northern blots**

Total RNA was isolated using the RNA-Zol® method (Tel-Test, Friendswood, TX) from 20 to 50 × 10^{6} Th17 T cells that were not stimulated or stimulated for 4.5 h with PMA plus ionomycin (100 ng/ml and 1 μM, respectively) in the absence or presence of 10 μM U0126, U0124 (an inactive analogue of U0126), the Parke-Davis (Ann Arbor, MI) MEK inhibitor (PD 098059) (29), or the SmithKline Beecham (Philadelphia, PA) p38 inhibitor (SK&F 8602) (8). Northern blots were performed as described (30) using 10 μg of total RNA/lane.

**Cellular extracts**

Total cellular extracts were made from A.E7 or Th17 T cells (10–50 × 10^{6} cells/treatment) that were not stimulated or stimulated for 15 min at 37°C with PMA plus ionomycin in the presence or absence of 10 μM U0126. Following the 15-min incubation, T cells were pelleted and washed twice with cold PBS. The cells were then lysed in 100 μl of cold lysis buffer (31). After a 10-min incubation on ice, the extracts were spun for 15 min at 14,000 rpm in Eppendorf tubes at 4°C to pellet cellular debris. The supernatant was removed and protein concentrations determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). These extracts were used in kinase assays and Western blots.

**Western blots**

Cellular extracts from A.E7 or Th17 T cells were used in Western analysis, as described earlier (31). Briefly, 10 to 20 μg of protein/sample were analyzed by SDS-PAGE on 10% Tris-Tricine gels (Novex, San Diego, CA). Protein was electrotransferred to polyvinylidene difluoride membrane, blocked with a solution of PBS containing 5% milk and 0.1% Tween-20, and probed with a rabbit polyclonal phosphospecific Ab against ERK or p38 (New England Biolabs, Beverly, MA), followed by a peroxidase-conjugated goat anti-rabbit secondary Ab. Washes following incubation with Abs were done in PBS + 0.1% Tween-20. Bands were detected using the Luminol chemiluminescent detection reagents (New England Biolabs). Blots were exposed to autoradiographic film (DuPont) for 1 to 2 min for detection.

**Kinase assay**

JNK assays were performed using cellular extracts of Th1 cells that were not stimulated or stimulated in the presence or absence of the drug U0126, as described previously (31).

**T cell proliferation assays**

A.E7 or Th17 cells (2 × 10^{5}/well) were incubated with 5 × 10^{3} mitomycin C-treated B10.BR or BALB/c splenocytes plus varying concentrations of pigeon cytochrome c or PR8 Ag (28), or with 5 U/ml human rIL-2. In addition, some assays contained U0126 or an inactive analogue, U0124, to determine direct effects of MEK inhibition on T cell proliferation. Two days after culture initiation, each well was pulsed with 1 μCi of [3H]TdR (DuPont NEN, Boston, MA) and harvested the following day. The incorporation of [3H]TdR into DNA was quantitated on a Packard Matrix 96 direct beta counter without the use of liquid scintillation mixtures.

**Effects of U0126 on T cell proliferation and response to restimulation**

During the preculture, A.E7 or Th17 cells (5 × 10^{5}/well) were cultured in 24-well plates for 3 days with 5 × 10^{3} mitomycin C-treated B10.A or BALB/c splenocytes with or without 0.1 μM Ag. Some of the cultures also contained 10 μM U0126 as an inhibitor of proliferation. Controls included Th1 cells incubated with APC alone in the presence or absence of the drug and the positive control for anergy that consisted of Th1 cells incubated on immobilized anti-CD3 alone, as described below. Following the preincubation step, viable cells were enumerated by trypan blue dye exclusion to determine the extent of proliferation. The T cells were then isolated by density-gradient centrifugation on Histopaque 1077 and rested in medium alone for an additional 4 days. Following the rest period, A.E7 cells (1–2 × 10^{5}) were washed and restimulated with normal APC plus Ag or with exogenous IL-2 alone in 96-well plates, as described below under restimulation.

**Anergy induction**

During the preculture, A.E7 or Th17 T cells (1–3 × 10^{6}/plate) were incubated in 10-cm Falcon 3003 petri plates coated with 10 μg/ml purified anti-CD3 mAb (clone 145-2C11; Boehringer Mannheim) (32) to induce anergy, as previously described (33). Some of the induction cultures also contained 10 μM U0126 or the inactive analogue, U0124. T cells were removed from the immobilized anti-CD3 after 1 to 2 days, washed, and transferred into fresh wells, where they were allowed to rest in medium alone for 2 to 3 days before restimulation.

**Restimulation of T cells**

Following the rest period, Th1 cells were restimulated with APC plus Ag (for proliferation assays) or PMA plus ionomycin (100 ng/ml and 1 μM, respectively, for cellular extracts used in kinase assays) as a stimulus to detect differences between control resting T cells and anergic T cells.

**Mixed leukocyte reaction**

PBMC were isolated from normal human donors by centrifugation through Vacutainer cell preparation tubes with sodium heparin gel (Becton Dickinson, Franklin Lakes, NJ). Cells from two unrelated donors were cultured together at a final concentration of 2 × 10^{5} cells/well in 96-well plates at 37°C in 5% CO_{2}. Various concentrations of compound were added at the
initiation of culture. After 5 days, the plates were then pulsed with 1 μCi/well [3H]TdR and harvested after 16 to 18 h.

Cell cycle analysis

PBMC were stimulated for 72 h with 1 μg/ml Con A in the presence or absence of 10 μM U0126 or 1 μM cyclosporin A (CsA). In some cultures, exogenous IL-2 was added at 10 U/ml. The cells were then fixed with ethanol, stained with propidium iodide, and analyzed by flow cytometry.

Results

U0126 inhibits ERK activation without affecting the JNK and p38 pathways

U0126 (Fig. 1) was initially identified as an inhibitor of AP-1 activity, and was shown to specifically inhibit MEK in a direct enzyme assay (manuscript in preparation). To determine whether U0126 showed selective inhibition of MEK in T cells, cellular extracts from murine Th1 clones that were stimulated for 15 min with PMA plus ionomycin in the presence or absence of 10 μM U0126, respectively. Quantitation was performed using the Image Quant program. Numbers displayed in parentheses below the bands represent fold increase over unstimulated and are corrected for loading using SP-1 as a standard.

[Image 94x557 to 256x733]

FIGURE 2. U0126 inhibits ERK activation without affecting the JNK and p38 pathways. Extracts of A.E7 T cells were A, probed by Western blot with an Ab that recognizes the phosphorylated form of ERK; B, probed by Western blot with an Ab that recognizes the phosphorylated forms of p38; D, probed by Western blot with an Ab that recognizes SP-1; or C, examined by kinase assay for the ability of JNK to phosphorylate a recombinant c-Jun protein. Lane 1 represents unstimulated cells, and lanes 2 and 3 represent cells stimulated with PMA plus ionomycin in the absence or presence of 10 μM U0126, respectively. Quantitation was performed using the Image Quant program. Numbers displayed in parentheses below the bands represent fold increase over unstimulated and are corrected for loading using SP-1 as a standard.

As reported previously (10, 11) and as shown in Figure 2, all three MAP kinase pathways are up-regulated in T cells upon activation with PMA/ionomycin or anti-CD3/anti-CD28 cross-linking. We were therefore interested in determining what effect, if any, blocking only the ERK pathway would have on T cell proliferation and IL-2 production. Ag-specific T cell clones were stimulated with APC plus Ag or anti-CD3 plus anti-CD28 in the presence or absence of 10 μM U0126, and proliferation was measured by [3H]TdR incorporation after 3 days. As shown in Figure 3A, T cell proliferation was blocked significantly in the presence of U0126, whereas the inactive analogue, U0124, had no effect. Up-regulation of IL-2R during stimulation of this Th1 clone with APC plus Ag was not inhibited by U0126 (data not shown) and, as shown in Figure 3B, the T cell clones can proliferate if IL-2 is added exogenously to the culture. Therefore, blocking the ERK pathway inhibits T cell proliferation in response to TCR cross-linking, but is not inhibitory to cell cycle progression in general if mitosis is initiated by a method that bypasses TCR signaling. This experiment also establishes that U0126 is not inherently toxic to T cells.

U0126 inhibits proliferation and cell cycle entry of human PBLs

The experiments described above were conducted in murine T cell clones. Since there may be differences in the signaling events in freshly isolated cells, we also tested the effects of U0126 in an MLR using PBLs isolated from normal human donors. As shown in Figure 4, U0126 effectively suppressed an allogeneic response, having an IC50 value of 2 μM. U0126 also blocked the proliferative response of PBLs to Con A and anti-CD3 cross-linking (not shown).

We observed that addition of compound 24 h after stimulation had no effect on T cell proliferation (not shown). This suggested that we were blocking an early event in T cell activation. We therefore examined the effects of U0126 on cell cycle and could show that the PBLs do not progress past G0/G1 when activated in...
the presence of U0126 (Table I). However, in agreement with the studies in the murine Th1 clones, the PBLs can enter into cell cycle if IL-2 is exogenously provided. This result is similar to the effects observed with the known immunosuppressant, CsA.

**U0126 blocks T cell proliferation by decreasing IL-2 mRNA levels**

The results shown above suggest that T cell proliferation is blocked by U0126 because the cells fail to produce IL-2 since proliferation occurs if IL-2 is added exogenously. To determine whether T cell proliferation is blocked by U0126 at the level of IL-2 transcription, or at a later stage affecting IL-2 synthesis and/or secretion, we performed Northern analysis on cloned T cells activated by PMA plus ionomycin in the presence or absence of the drug. As shown in Figure 5, U0126 prevented the PMA/ionomycin-induced up-regulation of IL-2 mRNA levels by 87% at 10 μM (lane 3), indicating that U0126 blocks T cell proliferation by interfering with IL-2 production, as would be expected of an AP-1 suppressor. In contrast, the inactive analogue, U0124, the Parke-Davis MEK inhibitor PD 098059 (lane 5), or the p38 inhibitor SK&F 86002 (lane 6) did not affect IL-2 mRNA levels (lane 4) appreciably at 10 μM. However, treatment with the Parke-Davis MEK inhibitor at a 20 μM concentration did result in a 50% reduction of IL-2 levels in Jurkat cells (data not shown), indicating that it is less potent than U0126. This is consistent with the differences in their potencies against the MEK enzyme. U0126 has an IC50 of 70 nM, whereas PD098059 has an IC50 of 5 μM (data not shown). It should be noted that although U0126 treatment results in decreased IL-2 mRNA levels, these experiments do not distinguish between an effect on IL-2 transcription or mRNA stability.

**Blocking the ERK pathway blocks T cell proliferation without inducing anergy**

Previous studies have indicated that TCR occupancy in the absence of proliferation induces anergy in murine Th1 clones (34). In these early studies, signaling through the TCR was allowed to proceed to completion, resulting in IL-2 production. Proliferation was then blocked by interfering with IL-2R binding/signaling or by mechanisms that blocked entry into the cell cycle. It was therefore interesting to determine whether inhibiting proliferation by blocking ERK activation would also result in anergy, since we would in effect be preventing completion of TCR signaling into the nucleus. To study this, T cells were preincubated with APC plus Ag in the presence or absence of U0126 for 3 days, after which the drug was washed off and the T cells rested in medium alone for 4 days before antigenic restimulation. To rule out cytotoxicity, T cells were also incubated with APC plus drug in the absence of Ag. T cells exposed to immobilized anti-CD3 in the absence of costimulation were used as a positive control for anergy. Figure 6A shows that proliferation in response to APC plus Ag was blocked effectively by U0126 during the preincubation step. As shown in Figure 6B, Th1 cells preincubated with APC plus Ag in the presence of the MEK inhibitor showed a good restimulation response, equal to resting T cells. This finding suggests that to induce anergy, TCR signaling has to be completed, and that blocking the ERK pathway does not interfere with the ability of the T cells to respond to subsequent restimulation.

**Blocking the ERK pathway does not interfere with anergy induction**

To study the effects of a MEK inhibitor on anergy induction, Th1 cells were exposed to immobilized anti-CD3 with no costimulation in the presence or absence of U0126. After 2 days, the cells were removed from the anti-CD3, washed, and rested for 3 days before restimulation with APC plus Ag. As shown in Figure 7A, blocking the ERK pathway did not interfere with the induction of anergy. Th1 cells anergized in the presence or absence of U0126 showed much lower levels of proliferation during restimulation compared

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**Table I. PBLs stimulated with Con A in the presence of U0126 fail to enter cell cycle**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% G0/G1</th>
<th>% S</th>
<th>% G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>77.2</td>
<td>1.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Con A</td>
<td>62.3</td>
<td>20.2</td>
<td>10.0</td>
</tr>
<tr>
<td>Con A + U0126</td>
<td>73.3</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>71.7</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Con A</td>
<td>75.1</td>
<td>8.9</td>
<td>4.2</td>
</tr>
<tr>
<td>Con A + U0126</td>
<td>70.1</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Con A + CsA</td>
<td>65.0</td>
<td>0.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Con A + U0126 + IL2</td>
<td>74.3</td>
<td>8.6</td>
<td>4.9</td>
</tr>
<tr>
<td>Con A + CsA + IL2</td>
<td>75.6</td>
<td>4.6</td>
<td>3.3</td>
</tr>
</tbody>
</table>

*PBLs were stimulated for 72 h with 1 μg/ml Con A in the presence or absence of 10 μM U0126 or 1 μM CsA. In some cultures, exogenous IL-2 was added at 10 U/ml. The cells were then stained with propidium iodide and analyzed by flow cytometry. Data are the results of two independent experiments. Cells with sub-2N amounts of DNA were not gated out and comprised from 10 to 25% of the population.
with resting T cells. The cells were still viable, though, because they proliferated well in response to exogenous IL-2. In fact, they responded better to exogenous IL-2 than resting T cells (Fig. 7B) perhaps due to the fact that IL-2R had been up-regulated during incubation on anti-CD3. These results indicate that signaling through the ERK pathway is not a prerequisite for the induction of anergy.

Discussion

The relative importance of the ERK, JNK, and p38 pathways in T cell activation cannot be determined unless specific inhibitors that selectively block each of these pathways are available. In this study, we have used a compound that specifically inhibits signaling through the ERK pathway without significantly affecting either the JNK or p38 pathways to try to dissect out the effects of blocking this pathway on T cell activation and anergy. We have shown that the effects of blocking the ERK pathway translate to decreased proliferation by T cells exposed to APC plus Ag or cross-linked anti-CD3 plus anti-CD28 Abs, and that proliferation was blocked at the level of inhibiting steady state levels of IL-2 mRNA. Our results are in agreement with data that show that inhibiting ERK activity by expressing a dominant negative mutant of MEK decreased IL-2 promoter activity (35), and confirm that the ERK pathway is critical for IL-2 production. In contrast, use of a known inhibitor of the p38 pathway, SK&F 86002 (8), did not decrease IL-2 mRNA levels or Ag-induced proliferation to nearly the same extent (data not shown), suggesting that the p38 pathway may be less critical in TCR-mediated signaling. Unlike its effects on TCR-mediated signaling, U0126 did not block IL-2-induced proliferation. This suggests that IL-2R signaling does not rely exclusively on the MAP kinases, but occurs via another pathway, possibly the JAK/STAT pathway (36).

Clonal anergy is a state of long-lasting unresponsiveness to antigenic stimulation induced in Th1 cells as a result of TCR occupancy in the absence of proliferation. It is induced half maximally by 5 h and maximally after 16 h of TCR occupancy in the absence of costimulation (23). The anergic state involves Ca\(^{2+}\) increases within the cell since anergy can be induced by increasing intracellular Ca\(^{2+}\) (23) and is also blocked by CsA (33, 37). Anergy induction requires new protein synthesis (38) and appears to be a result of an IL-2 production defect since anergic cells can proliferate in response to exogenously added IL-2.
Because these features suggest that anergy results from an active signaling process, we were interested in examining the effects of a MEK inhibitor on anergy induction. We could show that blocking T cell proliferation via inhibition of ERK activation did not induce anergy. Cells stimulated with Ag in the presence of U0126 show a restimulation response identical to resting T cells or T cells activated in the absence of U0126, indicating that blocking the ERK pathway in and of itself does not result in anergy. In addition, blocking the ERK pathway did not prevent anergy induction following TCR occupancy in the absence of costimulation. These results suggest that in contrast to the critical role of the ERK signaling pathway in the events leading to IL-2 production, this pathway does not regulate the induction of anergy when the TCR is cross-linked in the absence of costimulation. The reason for this may be that the anergy program is set in motion at a point upstream or parallel to MEK, possibly at the level of Ca<sup>2+</sup> increases within the cell following receptor cross-linking. In support of this, it is known that CsA blocks the induction of anergy in Th1 cells (33). This is thought to be due to the fact that this drug abrogates Ca<sup>2+</sup> signaling by blocking translocation of the NF-ATp subunit into the nucleus following TCR ligation. NF-ATp then cannot complex with AP-1, bind to NF-AT sites, and up-regulate transcription of NF-AT-responsive genes. However, we have shown in this study that U0126, which blocks the PKC-induced arm of TCR signaling leading to the up-regulation of AP-1, does not affect the outcome of anergy induction. This finding is interesting because it suggests that anergy is not induced by a gene product that is regulated by binding of the NF-AT heterotrimer composed of the NF-ATp plus AP-1 subunits. If this were the case, then one would expect both CsA and a MEK inhibitor (by blocking NF-ATp and AP-1, respectively) to prevent anergy. Because only CsA blocked induction of anergy, it suggests instead that anergy is induced by either a Ca<sup>2+</sup>-responsive gene product or by a gene with an enhancer that is bound by the NF-ATp subunit alone. Characterization of the genes induced by TCR occupancy in the presence of a MEK inhibitor vs CsA will be of interest.

In these respects, U0126 belongs to a novel class of immunosuppressants that effectively block IL-2 synthesis and T cell proliferation without affecting the long-term outcomes of either T cell activation or tolerance. This may be of importance clinically. It has been reported that T cell-mediated autoimmunity results when the widely used immunosuppressive drug CsA is withdrawn following prolonged treatment (39), perhaps due to the fact that tolerance via clonal anergy cannot take place in the presence of CsA. In addition to inhibiting anergy induction in vitro, CsA has been shown to interfere with the deletion of thymocytes bearing self-reactive TCRs in vivo (40). U0126 is also distinct from immunosuppressants such as rapamycin that inhibit T cell proliferation by interfering with IL-2R signaling and induce T cell anergy (34). Although the simultaneous induction of anergy in the subset of T cells whose activation one is trying to block by the use of immunosuppressants is advantageous, other immune reactions are constantly ongoing. Agents that induce anergy nonspecifically may also lead to tolerance induction in useful immune responses that could become disruptive to the functioning of the immune system as a whole. Efforts to determine the viability of using a MEK inhibitor as an immune suppressant in vivo will therefore be important.

Acknowledgments

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References


