Conjunctival Fibroblasts Enhance the Survival and Functional Activity of Peripheral Blood Eosinophils In Vitro

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PURPOSE. To examine the effect of human conjunctival fibroblasts on the survival and functional activity of human peripheral blood eosinophils.

METHODS. Eosinophils were purified by negative immunoselection [magnetic activated cell sorter (MACS), purity > 97%] from volunteers with mild atopia. Fibroblasts were cultured from conjunctival specimens of healthy donors. Eosinophils were cultured on confluent monolayers of conjunctival fibroblasts or in culture medium alone. Eosinophil survival was evaluated by the trypan blue exclusion test. Eosinophil adherence was assessed by counting the attached cells after washing the cultures. Eosinophil viability and adherence in coculture were also assessed in the presence of anti-granulocyte-macrophage colony-stimulating factor (GM-CSF), anti-interleukin (IL)-3, and anti-IL-5 neutralizing antibodies. Cocultured eosinophils were activated by lipopolysaccharide (LPS) after 4 days in culture, and eosinophil peroxidase (EPO) release was determined as a marker of their activation.

RESULTS. Eosinophils cocultured with conjunctival fibroblasts had a significantly increased viability of 35.9% (P = 0.004) and 12.8% (P = 0.003) on days 4 and 8, respectively. Fibroblast-conditioned medium did not enhance the survival of eosinophils. The increase in eosinophil survival in coculture was partially inhibited by anti-GM-CSF (P = 0.019), anti-IL-3 (P = 0.033), or anti-IL-5 (P = 0.011), whereas eosinophil adherence was reduced by anti-GM-CSF alone (P = 0.034). LPS activation of eosinophils cultured for 4 days with conjunctival fibroblasts induced higher EPO release than in freshly isolated eosinophils (P = 0.01).

CONCLUSIONS. Human conjunctival fibroblasts induced prolonged survival and increased secretory function of human peripheral blood eosinophils. Increased survival is partially mediated by IL-3, IL-5, and GM-CSF. The coculture of conjunctival fibroblasts with eosinophils can serve as an in vitro system for the study of eosinophil behavior in the ocular surface and of cellular interactions in allergic eye diseases. (Invest Ophthalmol Vis Sci. 2000;41:1038–1044)

The ocular allergic inflammatory response involves a complex network of several inflammatory cells and mediators. These include antigen-presenting cells, T cells (mainly, Th2 type), mast cells, basophils, eosinophils, and fibroblasts. Eosinophils represent the main effector cells in allergic eye mechanism. Mast cells are normally found in the substantia propria of the normal conjunctiva. 1 Whereas mast cells are increased and activated in the allergic conjunctiva and largely contribute to the early-phase response of the allergic reaction, eosinophils are the main cell type recruited during the late phase and represent the main factor responsible for persistent inflammation and tissue damage, through their preformed and newly synthesized mediators. 2–3 Once in the tissues, eosinophils adhere, survive for several days, and release their mediators, which cause tissue damage mainly detected when the allergic inflammatory response becomes chronic. The cytokines responsible for the prolonged eosinophil survival are interleukin (IL)-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF), also termed eosinophil survival cytokines. 4–10 Moreover, it has been recently shown that eosinophils can contribute to the Th2-type cytokine profile of the allergic reaction through an active production of IL-4, IL-5, and GM-CSF. 4

Fibroblasts are also involved in allergic eye diseases, particularly in those manifesting persistent eosinophilic inflammation and giant papillae, such as vernal keratoconjunctivitis. 5 Moreover, fibroblasts are affected by inflammatory cells, and eosinophils have been shown to induce lung and skin fibroblast proliferation and to modulate their collagen production. 11,12 They represent not only target cells responding to inflammatory stimuli with tissue remodeling, but they may also contribute to modulation of allergic inflammation in view of...
their ability to respond to and produce various cytokines. In particular, fibroblasts have been shown to influence mast cell differentiation, survival, and functional activity, mainly by their ability to synthesize Stem Cell Factor and extracellular matrix components such as fibronectin and laminin. Studies have shown that fibroblasts are also able to enhance eosinophil survival in vitro. In fact, mouse 3T3 fibroblasts prolong human peripheral blood eosinophil survival in the presence of exogenous GM-CSF. Other studies have shown that human lung fibroblasts enhance eosinophil survival by their production of GM-CSF.

Relationships between eosinophils and fibroblasts are of great importance for understanding the mechanisms of persistence of allergic inflammation and tissue repair. Because eosinophils are found in increased numbers in various allergic eye diseases in the conjunctiva, in proximity to conjunctival fibroblasts, the interactions between these two cell types could resemble those that have been demonstrated to exist in lung and skin models.

The purpose of this study was to investigate the effect on eosinophil survival and functional activity of coculturing human peripheral blood eosinophils with human conjunctival fibroblasts. This coculture system could serve as an in vitro model to investigate the interactions taking place between eosinophils and fibroblasts in ocular allergic diseases.

METHODS

Fibroblast Isolation and Culture from Conjunctival Biopsy Specimens

This research followed the tenets of the Declaration of Helsinki. Conjunctival specimens were obtained by biopsy from the superior bulbar conjunctiva in four patients during cataract surgery, after they provided informed consent. None of these patients was treated with any topical drug during the month preceding the surgery. The specimens were cultured as explants in Dulbecco’s modified Eagle’s medium (DMEM, Biological Industries, Beith Haemek, Israel) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, 1 mM HEPES, and 10% fetal calf serum (FCS; Biological Industries) and incubated at 37°C in 5% CO₂. The outgrown fibroblasts were subcultured by treatment with 0.1% trypsin-0.02% EDTA (Sigma, St. Louis, MO) for 5 minutes. For coculturing with eosinophils, third-generation passaged fibroblasts were seeded in 96-well plates (Costar, Cambridge, MA) at 1 × 10⁴ cells per well in 300 µl DMEM-10% FCS.

Isolation and Purification of Eosinophils

Eosinophils were purified from four volunteers with mild atopia (16 to 48 years of age) who were not at the time taking any oral treatment for their condition and whose peripheral blood eosinophil counts ranged from 4% to 10%. Informed consent was obtained from the donors according to the guidelines established by the intramural Hadassah-Hebrew University Human Experimentation Committee. Eosinophils were purified from the peripheral blood as previously described. Briefly, venous blood (100–150 ml) was collected in heparinized syringes. Blood was subjected to dextran 6% (Sigma) sedimentation, and leukocytes were centrifuged on Ficoll-Paque (density, 1.077; Sigma) for 25 minutes at 700g at 4°C. Neutrophils in the granulocyte-enriched pellet were tagged with micromagnetic beads to anti-CD16 antibodies, lymphocytes were tagged with anti-CD3 antibodies, and mononuclear cells with anti-CD14 antibodies (Miltenyi, Biotech, Bergisch Gladbach, Germany). The tagged cells were then eliminated by passing them through a magnetic field (MACS). Eosinophils were collected at a purity of 97% to 100%, assessed by Kimura staining, and at a viability of more than 99%, assessed by trypan blue (Sigma) staining.

Coculture of Eosinophils with Conjunctival Fibroblasts

The medium of the fibroblast monolayers was gently aspirated, and eosinophils (1 × 10⁵ cells in 200 µl enriched medium (EM) consisting of RPMI-1640 [Biological Industries] supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, 1 mM HEPES, and 5% FCS) were added to each well of confluent monolayers of conjunctival fibroblasts (96-well plates) and incubated at 37°C in 5% CO₂ for up to 16 days. For extended periods of culturing, the culture medium was changed every 4 days by gentle aspiration and addition of fresh medium. Control samples consisted of eosinophils cultured in wells in the absence of fibroblasts. For all the experiments, the cocultures were repeated four times, each consisting of eosinophils and conjunctival fibroblasts taken from four different individuals.

To assess whether the increased survival of eosinophils cultured with fibroblasts was attributable to IL-3, IL-5, or GM-CSF, neutralizing mouse anti-human monoclonal antibodies to these cytokines (R&D Systems, Minneapolis, MN) were added to the fibroblast cultures when the eosinophils were added (day 0 of the experiments). Antibodies were added at a final concentration of 4 µg/ml each, found to be an optimal concentration from pilot experiments in which two antibody concentrations of 4 µg/ml and 40 µg/ml provided similar inhibition values. In addition, to validate the neutralizing capacity of the anti-cytokine antibodies, eosinophils were incubated with an optimal concentration of recombinant human (rh)GM-CSF, rhIL-3, and rhIL-5 in the presence of optimal neutralizing concentrations of, respectively, anti-GM-CSF, anti-IL-3, and anti-IL-5. A nonrelevant monoclonal antibody, human anti-CD3 antibody (Miltenyi) at a final concentration of 4 µg/ml, was added as a control in these experiments.

In some experiments, freshly isolated eosinophils were cultured with conjunctival fibroblast-conditioned medium. The conditioned medium was obtained by culturing confluent monolayers of conjunctival fibroblasts in DMEM-10% FCS for a period of 48 hours. To the eosinophils in 96-well plates (1 × 10⁵/200 µl EM) 200 µl conditioned medium was added, and the cells were cultured for 96 hours.

Assessment of Eosinophil Viability

To assess the viability of eosinophils under the various culture conditions, the trypan blue exclusion method was used as follows. To evaluate viable eosinophils in coculture with conjunctival fibroblasts, eosinophils were detached from the conjunctival fibroblast monolayer by treatment with trypsin for 3 minutes. In this way, fibroblasts remained adherent to the wells, while eosinophils detached from the fibroblast monolayer. Twenty microliters of the detached eosinophils was withdrawn from the wells by gentle resuspension. Trypan blue (20 µl) was added, and the cells were immediately inspected in
a hemocytometer under a light microscope. Samples were examined blindly by two different observers. The percentage of eosinophil viability was calculated as follows: % viability of eosinophils = (number of cells excluding the trypan blue as enumerated on the day of the test) \( \times \) 100/(number of cells seeded on day zero).

For eosinophils cultured in the absence of fibroblasts, 20 \( \mu l \) of the cells were withdrawn after gentle resuspension, and examination of cell viability was performed as described.

Assessment of eosinophil viability was performed after 4, 8, and 16 days of culture. The viability of eosinophils cultured in either conditioned medium or of eosinophils cocultured with fibroblasts in the presence of neutralizing antibodies was evaluated after 4 days of culture.

### Assessment of Eosinophil Adherence

The adherence of the eosinophils to the conjunctival fibroblast monolayers was assessed after 4 days of coculture. To assess adherence, wells were gently washed three times by the addition and aspiration of 300 \( \mu l \) EM. The remaining adherent eosinophils were counted under an inverted microscope, using a grided eyepiece, at \( \times 400 \) magnification in at least 14 different fields. The number of adherent eosinophils was calculated by multiplying the average number of cells per grid (counted along a diameter of the well) by the number of times the well area could be divided into the grid area. Data are given as percentage adherent eosinophils, which is calculated as follows: % adherence = \( n/10^6 \times 100 \), where \( n \) is the average counted cells per grid \( \times \) area of well/area of the grid.

In some experiments, the role of either GM-CSF, IL-3, or IL-5 in eosinophil adherence to fibroblasts was evaluated. Neutralizing antibodies to any one of these cytokines (4 \( \mu g/ml \)) were added to the eosinophil–fibroblast coculture on day 0, and adherent eosinophils were evaluated 4 days later. Cocultures were inspected under an inverted microscope on day 4, by three different observers, to account for individual variability. These experiments were performed in triplicate.

### Eosinophil Peroxidase Determination after Eosinophil Activation with Lipopolysaccharide

To assess their functional activity, eosinophils cocultured with conjunctival fibroblasts were incubated on day 4 for 20 minutes at 37°C with 1 \( \mu g/ml \) lipopolysaccharide (LPS, Sigma). Fibroblast monolayers in medium alone were also incubated with LPS (1 \( \mu g/ml \)), as well as freshly isolated eosinophils seeded in wells in the absence of fibroblasts immediately after purification (1 \( \times \) 10^5 cells/200 \( \mu l \) EM containing 1 \( \mu g/ml \) LPS). After incubation, supernatants were collected, centrifuged (5 minutes, 120g) and stored at −80°C until assessed. Eosinophil peroxidase (EPO) release was determined by a colorimetric assay, as previously described.\(^{22}\) The substrate solution consisted of 0.1 mM O-phenylenediamine dihydrochloride (Sigma) in 0.05 M Tris buffer (pH 8.0) containing 0.1% Triton X-100 (Sigma) and 1 mM hydrogen peroxide (Merck, Darmstadt, Germany). Aliquots (50 \( \mu l \)) of the different supernatants were incubated with 50 \( \mu l \) substrate solution for 10 minutes at room temperature. The reaction was stopped by the addition of 100 \( \mu l \) of 4 M sulfuric acid (BDH, Dorset, UK), and the absorbance was determined at 490 nm by spectrophotometer.

### Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using the unpaired Student’s t-test or the Mann-Whitney test where appropriate. \( P < 0.05 \) was considered significant.

### RESULTS

#### Effect of Conjunctival Fibroblasts on Eosinophil Viability

As can be seen in Figure 1, survival of eosinophils cultured in medium alone was 5.8% ± 2.8% and 0.52% ± 0.29% on days 4 and 8, respectively. Under these conditions, no viable eosinophils were detected on days 12 and 16. Addition of 50% fibroblast-conditioned medium to eosinophils did not affect their survival. In fact, on the fourth day, 3.56% ± 0.59% eosinophils were still alive in the conditioned medium. The viability of eosinophils cultured on fibroblast monolayers was increased to 29.9% ± 1.9% on day 4 (\( P = 0.004 \), Mann-Whitney test), to 12.8% ± 2.9% on day 8 (\( P = 0.003 \)), and to 3.4% ± 0.6% on day 12. On day 16, occasional eosinophils were still surviving (0.7% ± 0.04%; Fig. 1).

#### Effect of anti-6M-CSF, anti-IL-3 and anti-IL-5 Neutralizing Antibodies on Eosinophil Viability and Adherence in Coculture

The increased survival of eosinophils in coculture with conjunctival fibroblasts was partially inhibited by the addition of any one of these cytokines (4 \( \mu g/ml \)) to the coculture (Fig. 2). In fact, eosinophil viability on day 4 decreased from 29.9% ± 1.9% in coculture, to 11.8% ± 0.9% after addition of optimal concentrations of anti-GM-CSF antibodies (\( P = 0.019 \)), to 20.4% ± 1.4% with anti-IL-3 antibodies (\( P = 0.033 \)), and to 17.4% ± 0.7% with anti-IL-5 antibodies (\( P = 0.011 \)). The nonrelevant antibody anti-CD3 did not cause a significant change in eosinophil viability, compared with the eosinophils cultured in medium alone (25.5% ± 3.8%, \( P = 0.272 \)).

Eosinophils were found by morphologic criteria to adhere quickly to the conjunctival fibroblasts. In fact, they assumed a spindle shape instead of presenting their round refractile appearance (not shown). On day 4, 75.3% ± 4.8% of the eosinophils in coculture were found to be attached to the fibroblast monolayers, after extensive washing of the cultures. Incubation of the coculture with an optimal concentration of anti-GM-CSF antibodies decreased the adherence of the eosinophils to the conjunctival fibroblasts to 57.9% ± 4.2% (\( P = 0.034 \)). No significant changes in adherence were demonstrated when either anti-IL3 or anti-HL5 was added to the cocultures. The addition of anti-CD3 antibody to the coculture also did not affect eosinophil adherence (85% ± 7.4%, \( P = 0.286 \)).

### Eosinophil Functional Activity in Coculture with Conjunctival Fibroblasts

Next, we assessed whether eosinophils cocultured for 4 days with conjunctival fibroblasts were functionally active. Cocultures were incubated with the potent eosinophil stimulator, LPS, and the release of EPO was evaluated as a measure of eosinophil activation.

Freshly isolated eosinophils incubated with an optimal concentration of LPS (1 \( \mu g/ml \)) released EPO at levels of 0.51 ±
0.05 optic density (OD; at 490 nm; Table 1). Similarly, after 4 days of coculture with conjunctival fibroblasts, eosinophils released EPO at 0.47 ± 0.04 OD, indicating that the surviving eosinophils were functionally active. Fibroblasts cultured for 4 days in medium alone, after 20 minutes' incubation with LPS (1 μg/ml), expressed peroxidase activity of 0.21 ± 0.06 OD. The EPO levels from freshly isolated eosinophils cultured in medium alone was 0.11 ± 0.024 OD (Table 1).

To compare the levels of EPO released from activated fresh eosinophils cultured alone with that in activated eosinophils cocultured with conjunctival fibroblasts, the background peroxidase levels were subtracted in these two groups as follows (Table 1). Peroxidase levels found in activated fibroblasts cultured in medium alone were subtracted from the EPO levels found in the coculture in each experiment. The mean net EPO levels of eosinophils in coculture was calculated to be 0.26 ± 0.03 OD. Next, EPO levels found in nonactivated eosinophils, were subtracted from the EPO levels found in the activated eosinophils, in each experiment, giving a net value of 0.39 ± 0.07 OD. Because at day 4 only 29.9% of the initial eosinophils seeded on conjunctival fibroblasts were viable, the EPO levels for the cocultured eosinophils was calculated per 10^5 cells. Thus, EPO levels found in activated eosinophils cocultured for 4 days with conjunctival fibroblasts (0.87 ± 0.09 OD per 10^5 cells) was significantly higher than those secreted from activated freshly isolated eosinophils (0.39 ± 0.07 per 10^5 cells; P = 0.01, paired t-test).

**DISCUSSION**

In the present study, we have demonstrated that coculturing human peripheral blood eosinophils with human conjunctival fibroblasts results in prolonged survival, adherence, and enhanced functional activity of eosinophils. Extended eosinophil survival in tissues seems to be the key feature of allergic inflammation. In our coculture, 30% of the initially seeded eosinophils were viable at day 4, and 13% survived after 8 days in culture, whereas in medium alone only 5% were still alive at day 4. In addition, we investigated the role of three survival cytokines for eosinophils—namely, IL-3, IL-5, and GM-CSF—with this system. The addition of an optimal concentration of neutralizing antibodies to either one of these three cytokines significantly inhibited the increased survival of eosinophils cultured with conjunctival fibroblasts, indicating a role for the three of them.

Previous studies have shown increased survival of eosinophils when they are cultured with mouse 3T3 fibroblasts, human lung fibroblasts, and myofibroblasts from bronchial mucosa in the presence of GM-CSF. In these studies, the fibroblast-induced prolonged survival of eosinophils was attributable to exogenous GM-CSF and to the production of this cytokine by the fibroblasts. However, in our study, in addition to the role of GM-CSF, we found that IL-3 and IL-5 also contribute to increased eosinophil survival.
The effects of the three cytokines (IL-3, IL-5, and GM-CSF) on eosinophil survival in vitro was previously demonstrated by adding these cytokines to cultured eosinophils. Specifically, the addition of GM-CSF to eosinophils cultured with 3T3 fibroblasts was reported by Owen et al. They demonstrated an increased survival of eosinophils with GM-CSF alone, which was further increased when the combination of GM-CSF and 3T3 fibroblasts was tested.

The source of these three cytokines in our coculture system could be autocrine secretion of the eosinophils or expression by the conjunctival fibroblasts. Although GM-CSF is shown to be secreted from fibroblasts, the evidence for IL-3 secretion from fibroblasts is anecdotal, and no evidence currently exists for the ability of fibroblasts to secrete IL-5. In a recent ex vivo study on eosinophil survival in nasal polyps, IL-5 was identified as a major survival factor for eosinophils, and its source was located in lymphocytes, mast cells, and eosinophils. Currently, it is not known whether conjunctival fibroblasts are capable of producing any of the three cytokines studied. Several studies have demonstrated the presence of IL-3, IL-4, IL-5, IL-6, IL-13, and tumor necrosis factor-α in biopsy specimens of conjunctiva from patients with allergic eye disease. However, the source of these cytokines was related to mast cells, T lymphocytes, eosinophils, and conjunctival epithelial cells. Therefore, we can assume that in our system, GM-CSF could have been secreted from both fibroblasts and

**TABLE 1.** EPO Release from Eosinophils After LPS Activation

<table>
<thead>
<tr>
<th>Culture Type</th>
<th>Culture Time</th>
<th>Total Peroxidase Levels Measured</th>
<th>Net Levels</th>
<th>Corrected Levels per $10^5$ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>E in medium</td>
<td>Freshly isolated eosinophils</td>
<td>0.11 ± 0.02</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E + LPS</td>
<td>Freshly isolated eosinophils</td>
<td>0.51 ± 0.05</td>
<td>0.39 ± 0.07</td>
<td>0.39 ± 0.07</td>
</tr>
<tr>
<td>F + LPS</td>
<td>4 days</td>
<td>0.21 ± 0.06</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E + F + LPS</td>
<td>4 days</td>
<td>0.47 ± 0.04</td>
<td>0.26 ± 0.03</td>
<td>0.87 ± 0.09 ($P = 0.01$)</td>
</tr>
</tbody>
</table>

EPO levels were assessed in supernatants by an enzymatic-colorimetric assay. Data are expressed in OD units and are the mean ± SEM of four different experiments performed in quadruplicate. LPS activation: 1 μg/ml, 20 minutes at 37°C. E, eosinophils; F, fibroblasts.

* (E + LPS) − (E in medium).
† (E + F + LPS) − (F + LPS).
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eosinophils, whereas the secretion of IL-5 and IL-3 could be an autocrine contribution by the eosinophils alone, stimulated by their contact with the fibroblast monolayers.

It is important to point out that other factors may be involved in the survival of eosinophils cocultured with conjunctival fibroblasts. In fact, an optimal concentration of anti-IL-3, anti-IL-5, and anti-GM-CSF neutralizing antibodies did not completely inhibit the survival of eosinophils. It is therefore conceivable that other mediators, produced by the fibroblasts and the eosinophils, such as tumor necrosis factor-α,286 stem cell factor,30 fibronectin, and laminin,30,31 could also contribute to this effect.

Interestingly, we could not demonstrate an increased viability of eosinophils when cultured with fibroblast-conditioned medium. The absence of effect of the conditioned medium is in agreement with previous studies6,10 but in contrast with another.8 The absence of effect of the conditioned medium implies that adhesion is necessary for both eosinophils and fibroblasts to secrete one or more of the three survival cytokines. Adhesion of eosinophils to tissue fibronectin has been shown to result in a significant autocrine production of GM-CSF, but not of IL-5,30 and blocking of β1 integrin adhesion molecules on eosinophils reduced their survival in coculture.10

In addition to the increased survival of eosinophils, coculture with conjunctival fibroblasts enhanced their functional activity after 4 days in culture, when compared with that of freshly isolated eosinophils. In fact, it was found that EPO release was suggested.33

In our coculture, eosinophils adhered to the fibroblast monolayers. This adherence was reduced by addition of anti-GM-CSF but was unchanged after the addition of either anti-IL-3 or anti-IL-5 to the system. It has been previously shown that both GM-CSF and IL-5 induce adhesion molecule expression on eosinophils and their adherence to endothelial cells.28 However, a number of studies point out a central role for GM-CSF secreted by human lung fibroblasts in increasing the activity of eosinophils as manifested by increased CD11b and decreased L-selectin expression. A combined mechanism of cell contact and cytokine release was suggested.33

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In conclusion, we have shown that coculture of human peripheral blood eosinophils with conjunctival fibroblasts influences eosinophil survival, adherence, and activation, and that GM-CSF is a key modulator of these effects. The increased viability and functional activity of eosinophils cocultured with conjunctival fibroblasts is probably a result of cellular contact and adhesion combined with cytokines released from both cell types as a result of this contact. This coculture can serve as an in vitro model for the study of eosinophil–fibroblast interactions in allergic eye diseases, especially vernal keratoconjunctivitis, in which a persistent eosinophilic inflammation is combined with intense fibroblast proliferation. In addition, the methodology used in this work may serve as a useful model for a better understanding of the relationship between eosinophils and fibroblasts in other allergic diseases, such as asthma, characterized by eosinophil infiltration and tissue remodeling.

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References


