Periductal Area as the Primary Site for T-Cell Activation in Lacrimal Gland Chronic Graft-Versus-Host Disease

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PURPOSE. To examine immune processes in the lacrimal gland of patients with chronic graft-versus-host disease (cGVHD) by evaluating the expression of surface molecules associated with T-cell activation.

METHODS. Antibodies to CD4, CD8, CD34, CD40, CD54, CD80, CD86, CD154, and HLA-DR were used for immunohistochemical analysis of lacrimal gland biopsy specimens obtained from nine patients with cGVHD and five with Sjögren's syndrome (SS). The regions of interest were further assessed by transmission electron microscopy.

RESULTS. CD4+ and CD8+ T cells were mainly detected in the periductal areas of the glands of patients with cGVHD, but were distributed throughout the acinar areas in patients with SS. In the periductal areas of patients with cGVHD, a subpopulation of CD4+ and CD8+ T cells expressed the activation marker CD154. In addition, CD4+ and CD8+ T cells were colocalized with mononuclear infiltrates and stromal fibroblasts expressing the full component of surface molecules necessary for antigen presentation, including HLA-DR, CD54, CD40, CD80, and CD86. Electron microscopy revealed activated fibroblasts that embraced lymphocytes and macrophages with their processes. Also, there were more CD8+ T cells in the glandular epithelia of patients with cGVHD than in those with SS. Intraepithelial T cells were attached to epithelial cells by several primitive contacts and colocalized with dead cells.

CONCLUSIONS. The results strongly suggest that CD4+ and CD8+ T cells in the lacrimal glands of patients with cGVHD are primarily activated in the periductal area through antigenic stimulation by potent antigen-presenting cells and stromal fibroblasts, and exert various effector functions, including cytotoxic effects on glandular epithelial cells. (Invest Ophthalmol Vis Sci. 2003;44:1888–1896) DOI:10.1167/iovs.02-0699

Hematopoietic stem cell transplantation (SCT) is now an established treatment for various hematologic malignancies.1,2 Because the number of long-term survivors who have undergone allogeneic SCT is increasing, prophylaxis and treatment of chronic graft-versus-host disease (cGVHD) have become important clinical issues. The eye, mouth, liver, lung, skin, and intestine are preferential targets of cGVHD.3 Dry eye associated with cGVHD is one of the major late complications after allogeneic SCT, having a significant impact on patients’ quality of life4,5 and sometimes leading to blindness6. We have reported that approximately half the patients who undergo SCT experience development of dry eye 6 months later. Severe dry eye resembling Sjögren’s syndrome (SS) progresses rapidly after the onset of symptoms in most of these patients.7 Although several supportive treatments are currently used to minimize symptoms in patients with lacrimal gland cGVHD,7–9 specific therapies have not yet been established.

The pathogenesis of cGVHD was originally explained as an alloimmune response to the recipient cells by donor lymphocytes,10 but the detailed mechanisms of its progression remain unclear. Our recent histopathologic study of the lacrimal glands of patients with cGVHD demonstrated prominent fibrosis and an increase in CD34+ stromal fibroblasts in the glandular interstitium, indicating a significant role for stromal fibroblasts in rapidly progressive dry eye.11 We also noted that T cells mainly infiltrated the periductal areas, rather than the acinar areas, but the immune processes that induce T-cell activation in the lacrimal gland were still largely unknown.

In this study, we examined the expression of surface molecules associated with T-cell activation including HLA-DR and adhesion and costimulatory molecules, in lacrimal gland biopsy specimens from patients with cGVHD. The activation status of the T cells that had infiltrated the lacrimal gland was also evaluated by assessing the expression of CD154, a marker for activated T cells.12,15

METHODS

Patients and Control Subjects

Lacrimal gland specimens were obtained by biopsy from nine patients with cGVHD (patients 1–5 and 11–14) and five patients with SS. Of these participants, five of the patients with cGVHD (1–5) and all the patients with SS contributed samples that were analyzed in our previous report.11 The diagnosis and classification of cGVHD were based on previously reported criteria.14 Briefly, patients with cGVHD were classified as having limited disease when they had localized skin and/or hepatic involvement alone and as having extensive disease when either generalized skin involvement or localized skin involvement with multiple organ involvement was present.14 All the patients with SS satisfied the diagnostic criteria proposed by Fox and Saito.15 Clinical characteristics of the nine patients with cGVHD are summarized in Table 1. At the time of biopsy, six patients (3–5 and 11–13) had severe dry eye, as defined by the Schirmer test with nasal stimulation of 10 mm or less,16 whereas the degree of dry eye in the remaining three patients was mild. Dry eye was the only clinical symptom related to cGVHD in two patients (4 and 14), but the remaining seven patients had dry eye as part of systemic cGVHD. Either cyclosporin A or tacrolimus in combi-
nation with methotrexate was used for posttransplantation GVHD prophylaxis. This regimen was discontinued approximately 6 months after transplantation in cases in which GVHD had not occurred before this time point. None of the patients received cyclosporin A topically.

Written informed consent was obtained from all patients, in accordance with the tenets of the Declaration of Helsinki.

### Immunohistochemistry

A portion of each dissected specimen was immediately embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Inc., Elkhart, IN) and snap frozen in precooled isopentane at optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Inc., Elkhart, IN) and snap frozen in precooled isopentane at —80°C. The remainder of the lacrimal gland tissue was fixed in 10% neutral buffered formalin and embedded in paraffin wax. Immunohistochemical analysis was performed according to a standard protocol.\(^{17-20}\) with a panel of mouse monoclonal antibodies to CD4, CD8, CD34, CD40, CD54, CD80, CD86, HLA-DR, and CD154 (Table 2). Frozen sections were used for the staining of all surface molecules, whereas paraffin-embedded sections were used only for the CD34 staining. Briefly, consecutive 5-μm-thick frozen sections were air dried, fixed in acetone for 20 minutes at room temperature, and rehydrated in phosphate-buffered saline (PBS). Nonspecific binding was inhibited by incubating the specimens with 5% rabbit serum in PBS for 30 minutes at room temperature. The sections were incubated with the optimally diluted primary antibody at room temperature for 2 hours, followed by incubation with a peroxidase-conjugated rabbit anti-mouse IgG antibody (Dako, Glostrup, Denmark) for 45 minutes. The bound antibodies were visualized by the addition of diaminobenzidine tetrahydroxychloride. All steps were followed by three washes with PBS. Nuclei were counterstained with hematoxylin for 1 minute. A negative control section without the primary antibody was prepared for each tissue specimen. To confirm that an adequate region was examined for immunohistochemistry, the first and last consecutive sections of each specimen were stained with hematoxylin and cosin.

In some experiments, acetone-fixed tissue sections were double-stained with a phycoerythrin-conjugated anti-CD154 antibody (Immunotech, Marseille, France) and a fluorescein isocyanate (FITC)-conjugated anti-CD4 or anti-CD8 antibody (Ancell, Bayport, MN). In addition, coexpression of HLA-DR and CD40, CD80, or CD86 on stromal fibroblasts was examined by double staining with an FITC-conjugated anti-HLA-DR antibody (Sigma, St. Louis, MO) and a mouse anti-CD40, -CD80, or -CD86 antibody (Ancell) in combination with a tetramethylrhodamine isothiocyanate isomer R-conjugated rabbit anti-mouse secondary antibody (Dako). Isotype-matched fluorescein-conjugated mouse antibodies were used in control experiments. These sections were mounted and examined with a fluorescence microscope (Eclipse E800; Nikon, Tokyo, Japan) or confocal microscope (LSM5 Pascal Vario 2GB; Carl-Zeiss, Göttingen, Germany).

### Table 1. Clinical Characteristics and Expression of HLA-DR, CD54, and Costimulatory Molecules on Stromal Fibroblasts in Specimens from Patients with cGVHD

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age at Biopsy</th>
<th>Sex</th>
<th>Degree of Dry Eye</th>
<th>Donor Type</th>
<th>Time of Biopsy after SCT (mo)</th>
<th>GVHD Prophylaxis</th>
<th>cGVHD Lesions Other Than Eye</th>
<th>HLA-DR</th>
<th>CD54</th>
<th>CD40</th>
<th>CD80</th>
<th>CD86</th>
<th>Expression on Lacrimal Gland Stromal Fibroblasts*</th>
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<td>1</td>
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<td>F</td>
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<td>CyA</td>
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<td>+</td>
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<td>3</td>
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<td>F</td>
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<td>Tacrolimus</td>
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<td>+</td>
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<td>4</td>
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<td>+</td>
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* Determined by immunohistochemistry.

Fibroblasts were defined as having spindle-shaped morphology with oval-shaped nucleus located in the extracellular matrix. TBI, total body irradiation; CyA, cyclosporin A.
Transmission Electron Microscopic Examination
A portion of lacrimal gland tissue was immediately fixed with 2.5% glutaraldehyde and subjected to electron microscopic examination, as described previously. One-micrometer-thick sections were stained with methylene blue, and the portions of interest were then sectioned, and examined under an electron microscope (1200 EXII; JEOL, Tokyo, Japan).

Microscopic Analysis
The degree of CD4⁺, CD8⁺, and CD154⁺ cells infiltrating the lacrimal gland was graded according to the scale that was modified from the scoring method of Greenspan et al., which had five grades based on the number of mononuclear infiltrates and focuses. Specifically, the degree of cell infiltration was scored on a scale of 0 to 4 (grade 0, none; grade 1, 1–9 cells; grade 2, 10–49 cells; grade 3, 50–99 cells; and grade 4, 100 or more cells per 4-mm² section). Fibroblasts were morphologically identified as spindle-shaped cells with oval nuclei present in the extracellular matrix. The positive staining of HLA-DR, CD54, and costimulatory molecules on mononuclear cells was determined in comparison with mononuclear infiltrates in the same section as a positive reference. We regarded the expression as positive if CD34⁺ cells were predominantly CD4⁺ T cells, with a few CD8⁺ T cells, whereas CD8⁺ T cells, but not CD4⁺ T cells, were detected within the ductal and acinar epithelia. Similar findings were obtained from the remaining tissue samples from eight patients with cGVHD. Consecutive sections of a cGVHD tissue sample revealed CD4⁺ and CD8⁺ T cells colocalized with CD34⁺ stromal fibroblasts around a medium-sized duct (Figs. 1E–G). In contrast, in SS tissue specimens, CD4⁺ and CD8⁺ T cells were distributed in the acinar areas with many CD4⁺ CD8⁻ mononuclear cells (Figs. 2D, 2E). Interaepithelial CD8⁺ T cells were infrequently detected in SS specimens.

The degree of CD4⁺ and CD8⁺ T-cell infiltration was further assessed with a semiquantitative scoring method (Fig. 3). Infiltration of CD4⁺ T cells into the lacrimal gland was significantly less prominent in cGVHD than in SS (2.2 ± 1.1 vs. 3.8 ± 0.5, P = 0.01), although the degree of CD8⁺ T-cell infiltration was similar (3.7 ± 0.5 vs. 3.6 ± 0.9, NS). In addition, CD8⁺ T cells were present in the intraepithelial lesions in significantly more cGVHD than SS specimens (2.7 ± 0.5 vs. 1.0 ± 1.0, P = 0.001).

Activation Status of Infiltrating CD4⁺ and CD8⁺ T Cells
To evaluate the activation status of the T cells in the periductal area and intraepithelia, lacrimal gland specimens were examined for the expression of CD154, a ligand for CD40 expressed on antigen-presenting cells (APCs). CD154 is transiently expressed on CD4⁺ and CD8⁺ T cells through T-cell receptor-mediated activation, and it is therefore used as a marker for T cells recently activated by antigenic stimulation. CD154 expression was detected in lacrimal gland specimens from five of the nine patients with cGVHD and two of the five patients with SS, although the number of CD154⁺ cells was much less than the total number of CD4⁺ or CD8⁺ cells in all specimens (Figs. 2C, 2F; grade 1). CD154⁺ cells were mainly present in the glandular interstitium in both cGVHD and SS, but intraepithelial CD154⁺ cells were found exclusively in specimens from two patients with cGVHD (Fig. 2C). Further evaluation by double staining for CD4/CD154 or CD8/CD154 revealed that cells expressing CD154 in the periductal areas included both CD4⁺ and CD8⁺ T cells in patients with cGVHD (Figs. 2G, 2H), whereas the T cells expressing CD154 were exclusively CD4⁺ T cells in patients with SS (results not shown).

Distribution of Mononuclear Infiltrates Expressing HLA-DR, CD54, and Costimulatory Molecules
In consecutive sections of a representative lacrimal gland of a patient with cGVHD, mononuclear cells expressing HLA-DR and CD54 were detected around a medium-sized duct in conjunction with CD4⁺ and CD8⁺ T cells (Figs. 1E, 1F, 1H, 1D). Mononuclear cells expressing HLA-DR, CD54, CD40, CD80, or CD86 were detected in the glandular interstitium of all the patients with cGVHD and those with SS, although their distribution was different—that is, infiltration was mainly in the periductal area in the patients with cGVHD, whereas they were distributed throughout the intraglandular interstitium in pa-
Figure 1. Light microscopy of lacrimal glands from patients with cGVHD or SS. Hematoxylin and eosin staining (A) and CD34 immunostaining (B) of a lacrimal gland section from a patient with cGVHD involving severe dry eye (patient 5) show a markedly fibrotic interstitium, irregular cell loss of the acini at the hilus, and a large number of cells expressing CD34. (C) High-magnification view of (B) showing the spindlelike morphology of the CD34+ cells, consistent with fibroblasts. (D) CD34 immunostaining in an SS specimen showing a scarcity of CD34+ fibroblasts. Immunostaining of CD4 (E), CD8 (F), CD34 (G), HLA-DR (H), and CD54 (I) on consecutive sections of a lacrimal gland biopsy from a patient with cGVHD (patient 1). Cells expressing CD4, CD8, CD34, HLA-DR, and/or CD54 were colocalized around a medium-sized duct. Immunostaining of CD54, HLA-DR, CD40, CD80, and CD86 on lacrimal gland specimens from patients with cGVHD (J–N) and patients with SS (O–S). (L, M, N, arrowbeads) Stromal fibroblasts expressing the molecules of interest. (N, arrow) CD86+ mononuclear cells with dendrites. Coexpression of HLA-DR and CD40 (T), CD80 (U), or CD86 (V) on stromal fibroblasts in lacrimal gland specimens from patients with cGVHD detected by immunofluorescence double staining. Arrowbeads: fibroblasts expressing both HLA-DR and CD40, CD80, or CD86. H, hilus; D, duct; Ac, acini. Magnification: (A, B) ×110; (C) ×430; (D) ×190; (E–J, M–O, Q–S) ×150; (T–V) ×170; (L) ×620; (K, P) ×75.
FIGURE 2. Immunostaining of CD4, CD8, and CD154 on lacrimal gland specimens from patients with cGVHD or SS. Immunostaining of CD4 (A) and CD8 (B) in a cGVHD specimen (from patient 1) shows predominant CD8\(^+\) over CD4\(^+\) T-cell infiltration into the periductal areas and the presence of CD8\(^+\) T cells within the ductal and acinar epithelia (B, inset). CD154 staining (C) of a cGVHD specimen (from patient 12) shows the expression of CD154 on cells surrounding a medium-sized duct. Immunostaining of CD4 (D), CD8 (E), and CD154 (F) in an SS specimen (from patient 9) shows nearly equal numbers of CD4\(^+\) and CD8\(^+\) T cells in the acinar areas and the presence of CD154\(^+\) cells around the ducts. Immunofluorescence double staining of CD4 and CD154 (G) and CD8 and CD154 (H) on a cGVHD specimen (from patient 12).

Arrowbeads: cells expressing CD4 or CD8 and CD154. A, Acini; D, duct. Magnification: (A, B, D, E) \(\times 100\); (B, inset) \(\times 210\); (C, F) \(\times 200\); (G, H) \(\times 390\).
Expression of HLA-DR, CD54, and Costimulatory Molecules on Periductal Fibroblasts

In consecutive sections of lacrimal gland from a patient with cGVHD, CD40, CD80, and CD86 were detected in specimens from patients with cGVHD. In addition, fibroblasts expressing CD40, CD80, or CD86 were found in the glandular interstitium in patients with cGVHD (Figs. 1L–N, top portion of micrographs), although the fibroblasts expressing these costimulatory molecules were only a portion of the stromal fibroblasts. Some stromal fibroblasts coexpressed HLA-DR and CD40, CD80, or CD86 (Figs. 1T–V). Stromal fibroblasts expressing CD40, CD80, and CD86 in the lacrimal gland were detected in specimens from seven, five, and five patients with cGVHD, respectively. In contrast, stromal fibroblasts expressing HLA-DR, CD54, CD40, CD80, or CD86 were rarely detected in specimens from patients with SS (Figs. 1O–S).

When associations of the expression profiles of HLA-DR, CD54, CD40, CD80, and CD86 on stromal fibroblasts with the clinical characteristics were examined in nine patients with cGVHD, no significant association was found between the expression profile and the degree of dry eye, donor type, total body irradiation, timing of biopsy after SCT, prophylaxis regimen, or cGVHD lesions in organs other than the eyes (Table 1). However, it was noted that fibroblasts expressing all these molecules were observed in three of six patients with severe dry eye, but in none of three patients with mild dry eye.

Expression of HLA-DR, CD54, and Costimulatory Molecules on Glandular Epithelial Cells

HLA-DR was expressed on the ductal and acinar epithelial cells in lacrimal gland specimens from patients with cGVHD and/or SS, but its expression pattern was quite different in the two diseases. HLA-DR expression on ductal and acinar epithelia was focal in patients with cGVHD, especially in those with minimal abnormality in the lacrimal gland structure (Fig. 1K). In contrast, HLA-DR was homogeneously expressed on the ductal and acinar epithelial cells in patients with SS (Fig. 1P). Focal expression of CD54, CD40, CD80, and CD86 on the ductal and acinar epithelial cells was also observed in the gland sections from patients with cGVHD (Figs. 1J, 1L, 1M, 1N, top portion of micrographs), whereas glandular epithelial cells in sections from patients with SS expressed CD80 and CD86 (Figs. 1R, 1S), but not CD54 or CD40 (Figs. 1O, 1Q). When prevalence of the expression of HLA-DR, CD54, and costimulatory molecules on glandular epithelial cells were compared between patients with cGVHD and those with SS, CD40 expression was detected in six of nine patients with cGVHD, but in none of the five patients with SS (P = 0.035).

Electron Microscopic Analysis of the Periductal Area and Intraepithelial Lesions

The interaction between T cells and stromal fibroblasts in the periductal area was further investigated with electron microscopy in five patients with cGVHD, and representative findings are shown in Figure 4. An activated stromal fibroblast with well-developed rough endoplasmic reticulum appeared to surround a lymphocyte that was possibly a T cell (Fig. 4A). Stromal fibroblasts were also attached to other inflammatory cells, including macrophages and plasma cells (Fig. 4B). Similar findings were observed, mainly around the ducts, in specimens from all the patients with cGVHD.

Examination of the intraepithelial lesions with infiltrated lymphocytes in patients with cGVHD by electron microscopy revealed lymphocytes that were possibly T cells within the ductal epithelia attached to epithelial cells by several primitive contacts (Fig. 4C). Dead epithelial cells were occasionally observed adjacent to the infiltrating lymphocytes (Fig. 4D). Similar observations were made in tissue samples from three of five patients with cGVHD.

Discussion

In this study, the periductal area was the primary site for T-cell activation in the lacrimal gland in SCT recipients with lacrimal gland cGVHD. This conclusion is based on the accumulation of CD4+ and CD8+ T cells, CD154+ activated T cells, and mononuclear cells on stromal fibroblasts expressing HLA-DR, CD54, and costimulatory molecules, predominantly around the glandular ducts in lacrimal gland specimens from patients with cGVHD. The periductal area is also the site for increased stromal fibroblasts and excessive fibrosis in patients with cGVHD. A prominent infiltration of CD4+ and CD8+ T cells into the lacrimal gland was also detected in patients with SS,
but the infiltrating T cells were distributed throughout the acinar lesions.

We have reported that the degree of infiltration of lacrimal glands by T cells is similar between cGVHD and SS, with the predominant site of T cell infiltration being the periductal area in cGVHD and the acinar area in SS. The present study further evaluated the subsets of T cells infiltrating the lacrimal gland by immunostaining for CD4 and CD8. As a result, several differences were noted in cGVHD compared with SS, as follows. In the glands from patients with cGVHD (1) infiltrating CD4\(^{+}\) and CD8\(^{+}\) T cells were mainly concentrated around the ducts, (2) the degree of CD4\(^{+}\) T-cell infiltration was less prominent, and (3) intraepithelial CD8\(^{+}\) T cells were more frequently detected, compared with the glands from patients with SS. The predominance of CD8\(^{+}\) over CD4\(^{+}\) T-cell infiltration in the lacrimal gland of patients with cGVHD is consistent with the findings obtained from the lacrimal glands of animal models for cGVHD and from salivary glands in patients with cGVHD. A subset of CD4\(^{+}\) and CD8\(^{+}\) T cells in the periductal area expressed CD154 and was likely to represent cells that were recently activated on their recognition of allo- or autoantigenic peptides presented by functional APCs within the periductal area. The activated CD4\(^{+}\) T cells in the periductal area could exert multiple effects, including the induction of phenotypic changes in stromal fibroblasts and the activation of cytotoxic CD8\(^{+}\) T cells. The functional interaction between CD4\(^{+}\) T cells and fibroblasts may result in the proliferation and activation of fibroblasts through cell–cell contact and T cell–derived soluble fibrogenic factors, such as IL-4, IL-6, and -17. In contrast, CD8\(^{+}\) T cells, which are activated through antigen recognition and the helper function provided by CD4\(^{+}\) T cells, may infiltrate the ductal epithelia. Electron microscopic findings showing abundant primitive contacts between T cells and glandular epithelial cells and the presence of dead epithelial cells.
T-Cell Activation Site in Lacrimal Gland cGVHD

HLA-DR, CD54, and costimulatory molecules were also expressed on glandular epithelial cells in cGVHD and SS, but the expression profiles were quite different between these two diseases. Focal expression of HLA-DR, CD54, CD40, CD80, and CD86 was seen in cGVHD, whereas a diffuse expression of HLA-DR, CD80, and CD86 was seen in SS. In this regard, Hiroki et al.34 have reported that the HLA-DR expression on salivary gland epithelia is associated with lymphocyte infiltration in cGVHD, but not in SS. Moreover, a significant, positive correlation between the intensity of CD54 staining on the bile duct epithelium, CD8+ T-cell infiltration, and histologic bile duct damage have been reported in an animal model of cGVHD.44 Therefore, in cGVHD, it is likely that the HLA-DR expression on epithelial cells is a result of the CD8+ T-cell infiltration into lacrimal glands, given that an upregulation of HLA-DR on epithelial cells can be induced by IFN-γ produced by activated T cells.35 It is also possible that the expression of HLA-DR, CD54, and costimulatory molecules on glandular epithelial cells is associated with the conditioning regimen or an infection in cGVHD. In contrast, the expression of HLA-DR, CD80, and CD86 by lacrimal gland epithelial cells in SS was consistent with the previous studies on the salivary glands in SS.45,46 and may be due to an intrinsic abnormality of the glandular epithelial cells in this disorder.57

In summary, our results demonstrate that the periductal area is a central site for inflammatory and fibrotic processes in lacrimal gland cGVHD. Further studies investigating the immune process in the periductal area in cGVHD would be useful for clarifying the complex pathogenesis of cGVHD as well as for the development of therapeutic strategies for this disease.

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References
