Expression of the Chemokine Receptor CXCR3 and Its Ligand IP-10 During Human Cardiac Allograft Rejection

Michael Melter, MD; Andrea Exeni, MD; Marlies E.J. Reinders, MD; James C. Fang, MD; Gearoid McMahon; Peter Ganz, MD; Wayne W. Hancock, MD; David M. Briscoe, MD

Background—Chemokines play an essential role in regulating the infiltration of leukocytes into allografts in experimental models. Little is known of their expression or function after human cardiac transplantation. Methods and Results—We analyzed 169 sequential human endomyocardial biopsies by immunocytochemistry for infiltration by CD3+ T cells and the expression of the chemokine receptors CCR1, CCR3, CCR5, and CXCR3. In both cross-sectional and longitudinal analyses, the expression of each of the chemokine receptors correlated with the degree of CD3+ T-cell infiltration. In particular, the expression of CXCR3 was temporally and spatially associated with CD3+ T-cell infiltrates and correlated with the histopathological diagnosis of acute rejection (OR, 11.73 and 4.05, respectively; P<0.001). Of 7 patients followed up longitudinally for 1 year, 4 with consecutive biopsies developed intimal thickening by intravascular ultrasound. In these patients, there was a trend for persistent expression of CD3- and CXCR3-expressing infiltrates in the later part of the first posttransplant year. The chemokines eotaxin, IP-10, lymphotactin, MCP-1, Mig, RANTES, and SDF-1 were examined in an additional 35 biopsies by RT-PCR. Eotaxin, lymphotactin, MCP-1, Mig, and SDF-1 were present in both normal and rejecting biopsies. However, the CXCR3 ligand IP-10, which was rarely expressed in normal biopsies, was markedly induced in acute rejection (OR, 19.43; P=0.01).

Conclusions—The presence of CXCR3+ T cells and the CXCR3 ligand IP-10 within endomyocardial biopsies is strongly associated with acute rejection. The CXCR3–IP-10 interaction warrants consideration as a therapeutic target in the management of cardiac allograft recipients. (Circulation. 2001;104:2558-2564.)

Key Words: transplantation ■ chemokine ■ rejection

Multiple molecules, including families of adhesion molecules and chemokines, provide signals for the dynamic trafficking of leukocytes into allografts. Chemokines are proinflammatory cytokines that function as potent chemoattractants for leukocytes.1-3 They provide signals leading to selective recruitment of mononuclear cells at sites of inflammation that may be critical in the process of acute rejection. The chemokines are a large family that is divided into 4 subfamilies, CXC, CC, C (also called lymphotactin), and CX3C, depending on the position of the first 2 cysteine residues.2 Each subfamily has relatively selective chemoattractant properties for different leukocytes. Chemokines act on responsive leukocyte subsets through G-protein–coupled 7-transmembrane receptors. Currently, 5 receptors for CXC chemokines, 11 receptors for CC chemokines, and 1 receptor each for CX3C and lymphotactin have been identified.3 Most chemokines bind to >1 receptor, and almost all chemokine receptors recognize >1 ligand. This redundancy in function suggests a limited sensitivity of leukocytes to single chemokines and/or that the regulation of chemokine production contributes to the selectivity of leukocyte subset recruitment. Also, it is suggested that chemokine receptor expression on select leukocyte subsets may constitute a major regulatory element for the composition of the mononuclear infiltrates in different types of inflammatory responses.

Several studies have demonstrated that chemokines participate in the inflammatory response within allografts.3,4 Indeed, it would appear from studies performed in experimental animal models that dysregulation of any one of a series of chemokines or chemokine receptors can limit the rejection reaction.5-7 In particular, recent studies comparing the effects of targeting various chemokine/chemokine receptor pathways experimentally with the use of knockout mice and neutralizing monoclonal antibodies have demonstrated that the IP-10–CXCR3 chemokine-chemokine receptor pathway is most important in acute rejection of cardiac allografts.5,7 Further identification of chemokines and chemokine receptors after human transplantation will likely provide information on
whether these may be novel targets for therapeutic manipulation to limit rejection. In this study, we have analyzed the expression of 4 different chemokine receptors and 7 complementary chemokines in human cardiac allografts. Our findings are that CXCR3 and the CXCR3 ligand IP-10 are most significantly associated with acute rejection.

Methods

Patients

Chemokine Receptor Study

We collected 179 biopsies from 16 cardiac allograft recipients (2 female, 14 male) during routine visits to the transplant clinic during the initial posttransplant year. In total, 169 were of sufficient quality and included 105 biopsies with normal histology, 6 with evidence of ischemic injury, and 57 with acute rejection (27 with International Society of Heart and Lung Transplant [ISHLT] grade 1, 13 with ISHLT grade 2, and 17 with ISHLT grade 3). One biopsy had evidence of both ischemic injury and acute rejection (ISHLT grade 1). Biopsies were taken at a median time of 118 days after transplantation (range, 4 days to 1 year). The median age of the recipients was 52 years (range, 36 to 64 years) at the time of transplantation. In addition, a total of 120 consecutive biopsies from 10 of these patients (2 female, 8 male) were analyzed longitudinally on a per-patient basis. None of the patients died or lost the graft.

Chemokine Study

RNA was prepared from 44 randomly selected biopsies within 4 weeks of collection. Biopsies were taken at a median time of 2.4 years after transplantation (range, 6 days to 14.4 years) from recipients (4 female, 40 male). The age of the recipients ranged from 19 to 64 years (median, 49 years) at the time of transplantation. Three patients lost their allograft in a median of 225 days (range, 168 to 235 days) after the study biopsy. 0.7 to 14 years after the transplantation. One patient died with a graft failure 122 days after the study biopsy, 10 years after transplantation. Sufficient RNA was harvested from 35 biopsies for analysis of all chemokine genes. A total of 26 had normal histology; 9 (26%) had evidence of acute rejection.

The immunosuppressive regimen consisted of corticosteroids (125 mg every 8 hours for the first 3 days tapered over 6 months to a final maintenance dose of 0.1 mg · kg⁻¹ · d⁻¹), cyclosporine at doses to maintain whole-blood levels between 300 and 350 μg/L for the first posttransplant year, and azathioprine. ISHLT rejection grades ≥2 were treated with intravenous bolus methylprednisolone and/or with a 10-day intravenous course of OKT3 monoclonal antibody.

Tissue Collection and Analysis

Endomyocardial biopsies (EMBs) were taken on all patients after heart transplantation as part of routine posttransplant care. A random portion of 1 specimen was used as the study biopsy and was stored at −80°C for later immunocytochemistry or for RNA extraction. Diagnostic specimens taken at the same time were processed for clinicopathological analysis according to the standardized system formulated by the ISHLT. The protocol for tissue collection was approved by the Human Research Committee at the Brigham and Women’s Hospital, Boston, Mass.

Intravascular Ultrasound

Intravascular ultrasound (IVUS) was performed at baseline and at 1 year after transplantation as previously described. Images were acquired at 3 to 7 random sites along the artery of interest, and sites from both studies were matched by use of angiographic landmarks. IVUS analysis was performed on digitized images with offline software (TapeMeasure, INDEC). Measurements included the intimal index (II) [II=intimal area/(intimal area+luminal area)] and maximal intimal thickness (MT; greatest radial intimal thickness). Progression of intimal pathology between IVUS studies was defined as a mean delta (ΔII) ≥5% or MT ≥150 μm.

Antibodies

Murine monoclonal antibodies used for immunocytochemistry included anti-human CXCR3 (I6C), CCR1 (2D4), CCR3 (7B11), and CCR5 (2D7) (Millennium Pharmaceuticals); anti-human CD3 (Pan-T3: DAKO); and K16/16 (nonspecific mouse IgG, a gift of M. Gimbrone, Brigham and Women’s Hospital) as a negative control.

Immunocytochemistry

Immunocytochemistry was performed on 4-μm cryostat sections as previously described.10 Primary antibodies were diluted in PBS and incubated with the sections overnight at 4°C in a humidified container. Subsequently, specimens were incubated with a species-specific peroxidase-conjugated secondary antibody, counterstained in hematoxylin, and mounted in glycerol-gelatin. Control specimens were incubated in nonspecific mouse IgG and medium alone.

Scoring System

Cellular staining was scored by 3 independent observers (M.M., A.E., and D.M.B.) as follows: 0=no stain, 1=minimal rare individual cells, 2=1 focal infiltrate, 3=multipolar infiltrates, and 4=diffuse infiltration, as previously described.10 Minor differences in scores were resolved by conference.

RNA Extraction and RT-PCR

Total RNA was isolated from the frozen EMBs by homogenization with the Ultraspec RNA Isolation System (Biotecx Laboratories, Inc) according to the manufacturer’s instructions. cDNA was prepared and PCR was performed with standard techniques as described11,12 and the following specific primers:
lipotactin: sense, 5'-AGG GCC CCT CAT TCA TCA G-3'; antisense, 5'-TTC CTT GGA AAA TGG CTT TG-3'; IP-10: sense, 5'-AGA GGA GAA ACC TCT AGT CTC AGC-3'; antisense, 5'-CCT CTG TGT GGT CCA CAA TT-3'; lymphotactin: sense, 5'-CTG ATC CTG GCC CTC CCT CT-3'; anti-sense, 5'-GGC TTC GTG TGG ATC ATG TT-3'; MCP-1: sense, 5'-AGT CCA ACC AAG GTT TGT GC-3'; antisense, 5'-GGC TTC GGA GTG TTG GAA GC-3'; MIG: sense, 5'-TTA AAC AAT TTG CCC CAA GC-3'; antisense, 5'-CTG TGT TGA GGA GTG GGA GG-3'; RANTES: sense, 5'-ACC ATG AAG GTC TCC GCG-3'; antisense, 5'-TTG AGG TTC TGA AGC TCA GTA C-3'; SDF-1: sense, 5'-AAG ACA CAA GGA GTG TGC ATT GAC C-3'; antisense, 5'-AGG TAC AGG GCA TGG AGT AA-3'. PCR reactions were performed under the following conditions: 1 cycle at 94°C for 5 minutes, followed by 40 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. The last cycle was extended to 7 minutes at 72°C. mRNA extracted from peripheral blood mononuclear cells served as positive control. Positive control for RT-PCR was the amplification of β-actin (Stratagene), and negative controls were the amplification of non–reverse-transcribed mRNA. Amplified products were resolved by electrophoresis, and band densitometry was analyzed with an Alphalmager 2000 system (Alpha Inno-tech Corp). Bands with a negligible signal (area under each peak <200) were considered negative. Other bands were positive. Samples with a negligible β-actin signal were excluded from analysis.

Statistical Analysis

Analyses were performed with SPSS for Windows 9.0. Logistic regression analysis was used to estimate the statistical significance of the relationship between categories. All results are presented as ORs with a 95% CIs. Values of P<0.05 were considered statistically significant. Correlation was analyzed by the Kendall’s tau-b test and was considered as significant when P<0.05.

Results

Chemokine Receptor Expression in Normal Human Cardiac Allograft Biopsies

A total of 169 EMBs were analyzed by immunocytochemistry for the expression of CD3+ T-cell infiltrates and for the expression of the chemokine receptors CCR1, CCR3, CCR5,
and CXCR3. Of these, 105 (62%) were histologically normal with no evidence of ischemic injury or allograft rejection. Although most normal biopsies had no infiltrates, others had evidence of isolated cells expressing CCR1, CCR5, and CXCR3 (Table 1). CXCR3 localized to individual cells, and CCR1 localized to small foci of infiltrates and a few sparsely distributed cells. CCR5 staining was weak and was restricted to rare, isolated cells. In contrast, CCR3 was expressed diffusely on granules within the cytoplasm of multiple resident cells (large macrophage and dendritic-like cells) in many normal biopsies (Figure 1f). CD3⁺ T-cell infiltrates were also present in many (40%) of these normal EMBs as single sparse cells or as isolated infiltrates (scores generally ≤1).

Chemokine Receptors in EMBs With CD3⁺ T-Cell Infiltrates and Acute Rejection

In a cross-sectional analysis of all 169 biopsies, high grades of CD3⁺ T-cell infiltrates (scores ≥2) were strongly associated with the presence of rejection (OR, 5.08; P<0.001; Table 1), especially moderate to severe rejection episodes accompanied by myocyte necrosis (OR, 7.8; P<0.001). Figure 1 illustrates the pattern of chemokine receptor expression in rejecting biopsies. CCR1 and CCR5 were expressed

<table>
<thead>
<tr>
<th>CD3⁺ T-Cell Infiltrates (Score ≥2), Chemokine Receptor Expression (Score ≥2), and Histopathological Diagnosis of Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Rejection</td>
</tr>
<tr>
<td>(n=105)</td>
</tr>
<tr>
<td>CD3⁺</td>
</tr>
<tr>
<td>CXCR3⁺</td>
</tr>
<tr>
<td>CCR1⁺</td>
</tr>
<tr>
<td>CCR3⁺</td>
</tr>
<tr>
<td>CCR5⁺</td>
</tr>
</tbody>
</table>

Figure 1. Chemokine receptor expression in human cardiac allografts. Representative photomicrographs of expression of CD3 (a), CCR1 (b), CCR5 (c), CXCR3 (d), and CCR3 (e) in biopsies with evidence of rejection. f, Expression of CCR3 in granules of resident cells in normal allografts. CCR3 was expressed on 2 populations of cells: resident cells within noninfiltrated grafts and infiltrating mononuclear cells in biopsies with evidence of rejection. Magnification: ×200 (a through c) and ×400 (d through f).

Table 1), especially moderate to severe rejection episodes accompanied by myocyte necrosis (OR, 7.8; P<0.001). Figure 1 illustrates the pattern of chemokine receptor expression in rejecting biopsies. CCR1 and CCR5 were expressed
on focal infiltrating mononuclear cells (Figure 1b and 1c) but varied in intensity at sites of infiltrates. CCR1 was most intense at sites of focal infiltrates and was strongly associated with the degree of CD3+ T-cell infiltrates (OR, 8.42; \( P<0.001 \); Figure 2). The expression of CCR5 was more intense at sites of focal infiltrates and was strongly associated with all grades of acute rejection (OR, 4.05; \( P<0.001 \); Table 1).

### Longitudinal Analysis of Chemokine Receptor Expression

We also assessed the temporal expression of CD3+ T-cell infiltrates and chemokine receptors in consecutive EMBs in 10 cardiac allograft recipients (median, 12 biopsies per patient; range, 11 to 14). Overall, infiltration by CXCR3-, CCR1-, and CCR5-expressing cells increased and decreased temporally in association with CD3+ T-cell infiltrates. Figure 4 illustrates the pattern of CD3+ T-cell infiltrates and CXCR3-expressing cells in EMBs from 2 representative patients over the period of the study. One patient (Figure 4A) had 8 biopsies with evidence of rejection, 7 of which were associated with myocyte necrosis (5 were ISHLT grade 3, 2 were grade 2, and 1 was grade 1 rejection). In this patient, each rejection episode was associated with infiltration of the EMB with multifocal/diffuse CXCR3+ expressing infiltrates (score \( \approx 2 \)) in association with CD3+ T-cell infiltrates. The other patient (Figure 4B) had 4 biopsies associated with rejection, albeit less severe (2 had ISHLT grade 2 and 2 had grade 1 rejection). CXCR3+ expressing infiltrates were again present in the EMB during each rejection episode, although the degree of infiltration was less marked with less severe rejection episodes.

The development of transplant coronary artery disease (TCAD) was evaluated in 7 patients by IVUS (Table 2). Four patients had evidence of an increased \( \Delta \text{MT} \) (median \( \Delta \text{MT} \), 300 \( \mu \text{m} \); range, 270 to 350 \( \mu \text{m} \)), whereas 3 patients had no change in intimal thickening between the baseline examination and 1-year study. Representative patients are illustrated in Figure 4. We analyzed whether persistent infiltration with both CD3+ and CXCR3 expressing infiltrates was associated with TCAD. Coinfiltration with both CD3+ and CXCR3+ expressing infiltrates (Figure 4C) occurred often in the first 6 posttransplant months. However, persistence of both CD3+ and CXCR3+ cells in biopsies in the third and fourth posttransplant quartiles was associated with the develop-

### TABLE 2. Median Overall Annual Scores for CD3+ T-Cell Infiltrates, Chemokine Receptors, and ISHLT Grade of Rejection: Association With the Development of TCAD

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD3</th>
<th>CXCR3</th>
<th>CCR1</th>
<th>CCR3</th>
<th>CCR5</th>
<th>Rejection</th>
<th>( \Delta \text{MT}, \mu \text{m} )</th>
<th>CMV†</th>
<th>CMV Rx†</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.A.</td>
<td>3 (1–4)</td>
<td>2 (1–3)</td>
<td>2 (0–4)</td>
<td>1 (0–3)</td>
<td>1 (1–3)</td>
<td>0 (0–3)</td>
<td>270</td>
<td>R+/D+</td>
<td>–</td>
</tr>
<tr>
<td>M.E.</td>
<td>3 (2–3)</td>
<td>2 (1–3)</td>
<td>1 (0–1)</td>
<td>2 (1–3)</td>
<td>3 (2–3)</td>
<td>1 (0–3)</td>
<td>310</td>
<td>R–/D+</td>
<td>–</td>
</tr>
<tr>
<td>C.O.</td>
<td>3 (1–4)</td>
<td>3 (2–4)</td>
<td>1 (0–4)</td>
<td>2 (0–3)</td>
<td>1 (0–3)</td>
<td>2 (0–3)</td>
<td>290</td>
<td>R+/D+</td>
<td>+</td>
</tr>
<tr>
<td>W.I.</td>
<td>1 (0–3)</td>
<td>2 (0–3)</td>
<td>1 (0–3)</td>
<td>2 (0–3)</td>
<td>2 (0–3)</td>
<td>1 (0–3)</td>
<td>350</td>
<td>R–/D–</td>
<td>–</td>
</tr>
<tr>
<td>A.U.</td>
<td>2 (1–3)</td>
<td>1 (0–3)</td>
<td>0 (0–3)</td>
<td>1 (1–3)</td>
<td>1 (0–3)</td>
<td>0 (0–2)</td>
<td>0</td>
<td>R+/D+</td>
<td>–</td>
</tr>
<tr>
<td>B.A.</td>
<td>1 (0–3)</td>
<td>1 (0–4)</td>
<td>0 (0–1)</td>
<td>0 (0–2)</td>
<td>1 (0–2)</td>
<td>0 (0–1)</td>
<td>0</td>
<td>R–/D–</td>
<td>–</td>
</tr>
<tr>
<td>B.O.</td>
<td>1 (0–3)</td>
<td>2 (0–3)</td>
<td>0 (0–2)</td>
<td>2 (1–3)</td>
<td>1 (1–3)</td>
<td>0 (0–2)</td>
<td>0</td>
<td>R+/D?</td>
<td>–</td>
</tr>
</tbody>
</table>

Scores range from 0 to 4.
*Change in maximal thickness in the first posttransplant year.
†Recipients and donors were either cytomegalovirus (CMV) positive (R+ and D+) or negative (R– and D–) by serology. CMV therapy (Rx) was given to 1 patient (+) who became symptomatic.
opment of TCAD (R=0.65, P<0.001). These findings are consistent with our previous observations that persistent intragraft immunological events in the third and fourth posttransplant quartiles identify patients at an increased risk for the development of TCAD.13

Chemokine Gene Expression in EMBs With and Without Evidence of Rejection
We next evaluated the expression of the chemokines eotaxin, IP-10, lymphotactin, MCP-1, Mig, RANTES, and SDF-1 within EMBs. Because expression of chemokines is reliably assessed at the mRNA level, we collected an additional number of allograft biopsies prospectively so that mRNA expression could be examined from fresh biopsy samples. A total of 35 biopsies were analyzed. Overall, we found that the expression of IP-10 and RANTES was strongly associated with the histopathological diagnosis of rejection (OR, 19.43, P=0.01; and OR, 7.88, P=0.02, respectively; Table 3 and Figure 5A and 5B). In contrast, the expression of eotaxin, lymphotactin, MCP-1, Mig, and SDF-1 was not associated with rejection (Table 3 and Figure 5C). Intragraft expression of IP-10 and RANTES was most easy to detect because each gene was either absent or present in the study biopsies (as illustrated in Figure 5A). This contrasted the expression patterns of MCP-1 and Mig, which were rarely expressed at all, and the expression of eotaxin, lymphotactin, and SDF-1, which were present in most biopsies examined. Together, our findings of enhanced mRNA expression of the CXCR3 ligand IP-10 and the CCR1 ligand RANTES is consistent with a functional role for these chemokines with chemokine receptors in human cardiac allograft rejection.

Discussion
In this study, we found that the chemokine receptors CCR1, CCR3, CCR5, and CXCR3 are expressed in association with CD3+ T-cell infiltrates in human cardiac allografts. Further-

### TABLE 3. Chemokine Gene Expression and the Histopathological Diagnosis of Rejection

<table>
<thead>
<tr>
<th></th>
<th>No Rejection (n=26), %</th>
<th>Rejection (n=9), %</th>
<th>OR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>41</td>
<td>44</td>
<td>1.16</td>
<td>0.86</td>
</tr>
<tr>
<td>IP-10</td>
<td>29</td>
<td>89</td>
<td>19.43</td>
<td>0.01</td>
</tr>
<tr>
<td>Lymphotactin</td>
<td>58</td>
<td>44</td>
<td>0.59</td>
<td>0.49</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0</td>
<td>0</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Mig</td>
<td>4</td>
<td>11</td>
<td>3.12</td>
<td>0.44</td>
</tr>
<tr>
<td>RANTES</td>
<td>31</td>
<td>78</td>
<td>7.88</td>
<td>0.02</td>
</tr>
<tr>
<td>SDF-1</td>
<td>69</td>
<td>89</td>
<td>3.56</td>
<td>0.27</td>
</tr>
</tbody>
</table>
more, infiltration with CXCR3 cells and the expression of the CXCR3 ligand IP-10 are closely associated with the diagnosis of acute rejection. CCR1 was also expressed on infiltrates, and the CCR1 ligand RANTES was present in high levels in allografts undergoing rejection. These findings are likely of clinical value in the monitoring of patients after transplantation. Moreover, our findings may be of mechanistic significance in that both IP-10 and RANTES are potent chemoattractants for antigen-activated T cells.1,2,14,15 Studies using knockout mice have demonstrated the importance of CCR1 in mononuclear cell recruitment during the development of acute allograft rejection.5 Furthermore, our findings focused on the association between CXCR3 expression and acute rejection, we also found that the pattern of CXCR3 expression tended to be different in a small number of patients with and without TCAD.

Although we found constitutive expression of intragraft IP-10 to be generally low, levels were increased during acute rejection. We believe this finding to be of great pathophysiological significance because IP-10 has recently been shown to be functional in experimental models of allograft rejection. Wild-type mice have a limited ability to reject IP-10 knockout donor cardiac allografts, whereas IP-10 knockout mice reject allogeneic grafts at the same tempo as wild-type mice.7 This suggests that intragraft expression of IP-10, such as by a graft endothelial cell, is critical for the recruitment of leukocytes and the rejection process.

RANTES, which binds to CCR1, CCR4, and CCR5, has previously been demonstrated to be expressed in human kidney and lung allografts.12,18,19 In addition, RANTES has been reported to be upregulated at different times after transplantation.20,21 And targeting CCR1 or CCR5 has been shown to suppress the development of acute and chronic rejection in a murine cardiac allograft model.5,22 Together, these data are consistent with a function for RANTES in the development of rejection. To this end, our data clearly demonstrate a correlation between the histopathological diagnosis of rejection and the expression of RANTES in human cardiac allografts. However, allografts in RANTES-knockout mice and allografts from RANTES-deficient mice are rejected normally.22 Hence, in contrast to the CXCR3–IP-10 pathway, the role of RANTES in vivo is likely to be in amplifying the alloresponse rather than as a requisite event in allograft rejection.

In summary, this is the first study to define the expression of chemokine receptors and chemokines in human cardiac allografts. Our findings are consistent with recent studies performed in animal models and are suggestive of a close association between the expression of CXCR3 and its ligand IP-10 with CD3 T-cell infiltrates and acute cardiac allograft rejection. Persistence of CXCR3 expression on infiltrates suggests that this molecule may play a functional role in the rejection of human cardiac allografts.

Acknowledgments

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References
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