Search for Immunomodulatory Effects of Blood Transfusion in Gastric Cancer Patients: Flow Cytometry of Th1/Th2 Cells in Peripheral Blood

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Abstract. Allogeneic transfusion seems to drive the immune system toward a Th2 response and away from a Th1 response, providing a hypothetical mechanism for transfusion-induced immunomodulation. By means of an intracytoplasmic cytokine detection technique with flow cytometry, it is possible to measure Th1 and Th2 cells derived from peripheral blood mononuclear cells. This study evaluated the presence of transfusion-induced immunomodulation in 11 gastric cancer patients after gastrectomy with perioperative blood transfusion, compared to 11 gastric cancer patients who were treated by gastrectomy without transfusion. Lymphocytes subsets, including CD4 T cells, CD8 T cells, CD4/CD8 Ratio, CD2(+) T cells, CD3(+) T cells, and CD19(+) B cells, were measured in these patients, as well as variables that might suggest transfusion-induced immunomodulation, such as duration of antibiotic use, duration of hospital stay, and total hospital charges. This study also measured changes in the Th1/Th2 ratio. Th1 and Th2 lymphocytes were characterized by measuring intracellular expression of cytokines with flow cytometry. Cells were stimulated with phorbol myristate acetate and ionomycin in the presence of brefeldin-A. The results showed no significant differences in lymphocyte subsets, Th1/Th2 ratio, total hospital charges, or duration of antibiotic utilization between the groups of transfused and non-transfused gastric cancer patients after gastrectomy. The only significant difference was a longer hospital stay for transfused patients (mean 20.5 da) compared to non-transfused patients (mean 16.2 da). The anticipated finding of a Th2 response after blood transfusion was not observed. A larger group of patients may be needed to document such an effect, since many confounding variables affect the morbidity and outcome of surgery in these patients.

Keywords: Transfusion-induced immunomodulation, Th1 cells, Th2 cells, gastric carcinoma, flow cytometry

Introduction

Allogeneic blood transfusion generally causes up-regulation of humoral immunity, with increased formation of alloantibodies to HLA-A,B antigens (MHC class I), and down-regulation of cellular immunity, with decreased cutaneous delayed type hypersensitivity, T cell proliferation, and natural killer cell function [1-6]. These changes have been suggested as underlying mechanisms for the various immunomodulatory effects of blood transfusion that have been reported, including (1) prolonged survival of organ allografts [7], (2) increased rates of cancer recurrence [6,8-9], (3) increased prevalence of postoperative bacterial infections [10-14], (4) reduced rates of repetitive spontaneous abortion [15], (5) improvement in Crohn's disease and rheumatoid arthritis [16], and (6) more rapid progression of chronic viral infections.

Blood transfusion has been suggested as the single most important contributing cause of postoperative bacterial infection [17]. Post-operative infection is about 4x higher (ie, 20%) in patients receiving allogeneic transfusions, compared to non-transfused...
patients (≤5%) [10-11]. In contrast to allogeneic transfusions, autologous transfusions are associated with rates of postoperative infection rates that are identical to those in non-transfused patients [18]. This suggests that the effects of allogeneic transfusion are causal and immunologically mediated [3].

Allogeneic transfusions are associated with a longer period of antibiotic use, longer hospital stays, and higher hospital costs, owing to increased postoperative infection and morbidity, while recipients of autologous transfusions or leukocyte-depleted allogeneic transfusions have morbidity rates and hospital costs that are comparable to those of patients who receive no transfusions [13,19-20]. Incremental postoperative costs due to blood transfusions are estimated to range from $1,000 to $2,000 per unit of allogeneic blood administered [3,19].

Some epidemiological studies indicate that allogeneic transfusions are associated with 2- to 4-fold increase in lymphomas diagnosed during 5 to 10 yr post-transfusion [17,21]. Since lymphomas are the most prominent tumors in acquired defects of the immune system, this finding is consistent with the hypothesis that transfused patients develop a mild to moderate deficiency of cellular immune function [3].

Recent investigations suggest that altered cytokine regulation may contribute to down-regulation of macrophage and T cell function and up-regulation of humoral immunity [1,3]. Allogeneic transfusion seems to drive the immune system toward a Th2 response and away from a Th1 response [1-4,21-24], providing an hypothetical mechanism for transfusion-induced immunomodulatory effects. This hypothesis is consistent with observations of post-transfusion immunomodulatory effects, such as increases in tumor recurrence, post-operative infection, and severity of viral infection, and decreases in recurrence of Crohn's disease [16], allograft rejection [2,8], and spontaneous abortion [15].

Using methods described by Sander et al [25] and modified by others [26-27], it has recently become possible to detect intracellular cytokines by fixation with paraformaldehyde, permeabilization with saponin, and subsequent indirect immunofluorescent staining with flow cytometry. The detection of intracellular cytokines by flow cytometry can differentiate the T helper subsets, Th1 and Th2 [26,28-31].

Gastric cancer has been recognized as the fourth leading cause of cancer deaths in Taiwan. The overall outcome for patients with gastric carcinoma has not significantly improved during the past decade [32]. For gastric carcinoma patients, perioperative allogeneic blood transfusion has been reported to have an adverse effect on immune responses, inducing higher risk of postoperative infection [32-34]. Although an association with poor survival of gastric cancer patients has frequently been reported, the independent influence of allogeneic blood on survival is still unproved [2-3,32]. However, as shown by meta-analysis, allogeneic transfusions carry a substantial risk (>2x increase) for immunomodulatory effects with impaired cellular immunity [35].

In this study, indicators of immunomodulation were evaluated in gastric cancer patients after allogeneic blood transfusion. Lymphocyte subsets, changes in the Th1/Th2 ratio, and a number of other variables that may be related to transfusion-induced immunomodulation, such as period of antibiotic use, length of hospital stay, and total hospital charges, were measured during the post-operative course of gastric cancer patients following gastrectomy.

Materials and Methods

Patients and controls. CD4 Th1 and Th2 subsets were measured in 25 healthy persons (without surgical operation) to establish normal reference ranges for the Taiwanese populace. Lymphocyte subsets including CD4, CD8, CD4/CD8 ratio, CD3(+) T cells, CD2(+) T cells, and CD19(+) B cells were measured in 284 healthy persons to establish normal reference ranges.

From September to December 1999, 35 patients with gastric cancer received gastrectomy as the primary treatment modality at the Chang Gung Memorial Hospital, Lin-Kou Medical Center (CGMH). Twenty-eight patients signed the consent form and agreed to have lymphocyte subsets measured during their post-operative courses. One patient with gastric lymphoma and another with a second cancer (of the lung) were excluded from the study. One patient refused to have lymphocyte subsets measured at 3 mo after gastrectomy. Two patients did not come for follow-up visits at the CGMH. One patient was excluded because of a blood transfusion at 5 da post-operation.
Overall, 22 gastric cancer patients were included in this study. The patients were divided into two groups according to their blood transfusion status. One group comprised 11 patients who received blood transfusion(s) during the operation or the first day after operation; the other group comprised 11 patients who were not transfused. Blood samples were collected from all patients at the beginning of the operation, at 4, 7, 14, 30, and 90 da after operation, and at intervals of 6 mo thereafter. The results obtained with the preoperative specimens served as the baseline values.

**Cell cultures.** The methods used for cell cultures here and in the intracellular cytokine staining section were

![Fig 1](image)

**Fig 1.** Intracellular staining of interferon-γ (IFN-γ) and IL-4 was performed after peripheral blood mononuclear cells were stimulated with PMA and ionomycin in the presence of BFA. The flow cytometry charts shown at the top of this figure were obtained with (panel B) and without (panel A) intracellular interferon-γ staining for Th1 cells; the charts shown at the bottom of this figure were obtained with (panel D) and without (panel C) intracellular IL-4 staining for Th2 cells.
described by Sander et al [25] and modified by Jung et al [26] and Rostaing et al [27]. Peripheral blood mononuclear cells (PBMCs) of normal persons and gastric cancer patients were isolated from heparinized blood specimens by ficoll gradient centrifugation (Ficoll-Paque Plus, Amersham Pharmacia Biotech, Uppsala, Sweden). The cells were harvested, washed, and suspended at a density of 1 x 10⁶ cells/ml in RPMI (GIBCO-BRL, Paisley, UK), supplemented with 5% heat-inactivated fetal calf serum (GIBCO-BRL), 50 µg/ml penicillin (GIBCO-BRL), and 50 µg/ml streptomycin (GIBCO-BRL). Cell suspensions (1 ml) were cultured in test tubes in the presence of 25 ng phorbol myristate acetate (PMA) (Sigma, St. Louis, MO), 10 µg brefeldin A (BFA)(Sigma), and 1 µg ionomycin (Sigma) at 37°C and 5% CO₂ for 4 hr.

**Intracellular stain.** Cultured cells (1 ml, containing 1 x 10⁶ cells), after washing with phosphate buffered saline (PBS, GIBCO-BRL), were suspended in 100 µl PBS and stained at room temperature for 30 min with surface Ab CD4-PerCP (Becton-Dickinson Immunocytometry Systems, BDIS, San Jose, CA). The cells were then fixed with fixation medium (FIX & PERM Cell Permeabilization Kit, CALTAG Laboratories, An Der Grub BioResearch, GmbH, Vienna, Austria) for 15 min at room temperature in the dark. After washing with PBS, Permeabilization Medium (FIX & PERM Cell Permeabilization Kit) was added, along with Interferon-γ FITC/Anti-Human IL-4 PE (BDIS). This mixture was incubated for 30 min at room temperature. The cells were washed with PBS and counted by a flow cytometer. As shown in Fig.1, intracellular staining of interferon-γ and IL-4 was possible after the PMBCs were stimulated with PMA and ionomycin in the presence of BFA. The Th1 and Th2 cells were thereby identified and the Th1/Th2 ratio was calculated.

**Immune monitor panel (lymphocyte subsets).** Cultured cells were adjusted to 5 x 10⁶ cells/ml with RPMI 1640 (GIBCO-BRL) and 5% FCS (GIBCO-BRL). Cell suspension (100 µl) was added. Then, mono Ab CD45 FITC/CD14 PE, normal control IgG1 FITC/IgG1 PE, CD2 FITC/CD38 PE, CD4 FITC/Leu8 PE, CD3 FITC/CD16+56 PE, and CD8 FITC/CD11b PE were added, respectively, and left at 4°C for 30 min. These reagents were all obtained from BDIS, San Jose, CA. After washing with PBS, 500 ml of 1% paraformaldehyde (Merck, Darmstadt, Germany) was added and the cells were counted by a flow cytometer.

**Flow cytometric analysis.** List mode data were acquired on a Coulter-EPICS-XL-MCL flow cytometer and analyzed using SYSTEM II software. Typically 10,000 events were acquired in the gating windows.

**Statistical analysis.** Statistical analyses were performed using Microsoft Excel 2000 programs. Within each group, the post-operative results were compared with the baseline values obtained on the day of operation. The values in the transfused group were also compared with the values in the non-transfused group at the same interval after surgery. The p values ≤0.05 by χ² test or Student’s two-tailed t test were considered significant.

**Results**

Of the 22 patients included in this study, one group comprised 11 patients who were transfused with 2 to 4 units of packed red cells. (In Taiwan, one unit represents the component prepared from 250 ml of whole blood.) Seven of these patients also received 4 units of fresh frozen plasma. None of the patients received platelet transfusion. Only patients who were transfused on the day of gastrectomy, or one day thereafter, were included in this group.

Demographic data for the groups of transfused and non-transfused patients are listed in Table 1. Except for different proportions of men to women, the groups of transfused and non-transfused patients showed no significant differences in regard to age, serum albumin, urea, and creatinine concentrations, serum aspartate aminotransferase activity, blood hemoglobin concentration, and leukocyte count (WBC), or the time that elapsed during the gastrectomy operation.

The data for the lymphocyte subsets, including CD19(+) B cells, CD2(+) T cells, CD3(+) T cells, CD4 T cells, CD8 T cells, and CD4/CD8 ratio, are listed in Table 2. There were no significant differences between the groups of transfused and non-transfused patients in respect to the lymphocyte subsets. Only the CD4/CD8 ratio at 30 da post-gastrectomy showed a marginal difference (p = 0.052). Also, in the
Table 1. Demographic data for gastric cancer patients who underwent gastrectomy, with or without perioperative transfusion. Laboratory tests were performed on serum (S) or blood (B) obtained at the beginning of the operation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Transfused patients (n = 11)</th>
<th>Non-transfused patients (n = 11)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M:F)</td>
<td>9:2</td>
<td>4:7</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>69.5 ± 17.5</td>
<td>60.3 ± 9.8</td>
<td>ns</td>
</tr>
<tr>
<td>S albumin (g/dL)</td>
<td>4.1 ± 0.4</td>
<td>4.0 ± 0.6</td>
<td>ns</td>
</tr>
<tr>
<td>S urea N (mg/dL)</td>
<td>17.3 ± 11.1</td>
<td>12.9 ± 6.1</td>
<td>ns</td>
</tr>
<tr>
<td>S creatinine (mg/dL)</td>
<td>1.12 ± 0.44</td>
<td>0.87 ± 0.19</td>
<td>ns</td>
</tr>
<tr>
<td>S AST activity (U/L)</td>
<td>17 ± 5</td>
<td>20 ± 5</td>
<td>ns</td>
</tr>
<tr>
<td>B hemoglobin (g/dL)</td>
<td>12.5 ± 1.3</td>
<td>12.2 ± 1.4</td>
<td>s</td>
</tr>
<tr>
<td>WBC (cells x 10^3/µl)</td>
<td>6.1 ± 1.6</td>
<td>6.9 ± 2.7</td>
<td>ns</td>
</tr>
<tr>
<td>Operation time (min)</td>
<td>283 ± 64</td>
<td>247 ± 35</td>
<td>ns</td>
</tr>
</tbody>
</table>

a mean ± SD
b ns = no significant difference between treatment groups

c Immunomodulatory effects of transfusion in gastric cancer

Table 3. Data for CD4 Th1/Th2 ratio and other indices that might be indicative of an immunomodulatory effect of perioperative transfusion in gastric cancer patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Transfused patients (n = 11)</th>
<th>Non-transfused patients (n = 11)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1/Th2 ratiob</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at operation</td>
<td>2.6 (1.6-3.7)</td>
<td>3.4 (1.6-6.5)</td>
<td>ns</td>
</tr>
<tr>
<td>4th day</td>
<td>4.0 (1.3-7.3)d</td>
<td>2.9 (1.0-5.5)</td>
<td>ns</td>
</tr>
<tr>
<td>7th day</td>
<td>2.8 (1.3-6.1)</td>
<td>2.9 (1.4-5.3)</td>
<td>ns</td>
</tr>
<tr>
<td>14th day</td>
<td>3.0 (1.3-6.3)</td>
<td>2.7 (1.7-4.0)</td>
<td>ns</td>
</tr>
<tr>
<td>30th day</td>
<td>2.8 (1.2-4.2)</td>
<td>3.7 (2.3-5.6)</td>
<td>ns</td>
</tr>
<tr>
<td>90th day</td>
<td>3.6 (2.1-6.1)</td>
<td>2.6 (1.2-4.7)</td>
<td>ns</td>
</tr>
<tr>
<td>Hospital stay (days)</td>
<td>20.5 (16-34)</td>
<td>16.2 (11-24)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Antibiotic use (days)</td>
<td>7.3 (2.1-5)</td>
<td>8.3 (2-19)</td>
<td>ns</td>
</tr>
<tr>
<td>Hospital charge (x 10^3)</td>
<td>133 (34-220)</td>
<td>118 (25-170)</td>
<td>ns</td>
</tr>
</tbody>
</table>

a mean (and range)

b Reference values for CD4 Th1/Th2 ratio in healthy Taiwanese subjects (n = 25) are mean 3.01, median 3.07, and range (1.89-4.06)
c ns = no significant difference between treatment groups
d p = <0.05 versus corresponding data at operation

c Immunomodulatory effects of transfusion in gastric cancer

Table 2. Lymphocyte subsets in gastric cancer patients after gastrectomy, with (n = 11) or without (n = 11) perioperative transfusion. Data are given as % of mononuclear cells. Reference values derived from healthy Taiwanese subjects (n = 284).

<table>
<thead>
<tr>
<th>Lymphocyte subset</th>
<th>Reference range</th>
<th>Treatment group</th>
<th>On day of surgery</th>
<th>4</th>
<th>7</th>
<th>14</th>
<th>30</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 T cells</td>
<td>38 (20-66)</td>
<td>Transfused</td>
<td>32 (5-62)</td>
<td>33 (11-60)</td>
<td>39 (6-61)</td>
<td>28 (17-53)</td>
<td>44 (40-49)</td>
<td>41 (25-55)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-transfused</td>
<td>31 (8-46)</td>
<td>39 (7-63)</td>
<td>45 (31-64)</td>
<td>40 (22-74)</td>
<td>38 (18-73)</td>
<td>39 (16-63)</td>
</tr>
<tr>
<td>CD8 T cells</td>
<td>27 (4-62)</td>
<td>Transfused</td>
<td>21 (4-39)</td>
<td>14 (1-27)</td>
<td>19 (3-38)</td>
<td>16 (1-42)</td>
<td>14 (13-19)</td>
<td>26 (16-34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-transfused</td>
<td>25 (12-39)</td>
<td>19 (5-46)</td>
<td>22 (6-37)</td>
<td>21 (9-41)</td>
<td>25 (9-35)</td>
<td>26 (7-54)</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>1.2 (0.6-2.2)</td>
<td>Transfused</td>
<td>1.4 (0.2-3.4)</td>
<td>2.7 (1.5-5.5)</td>
<td>2.0 (0.7-3.3)</td>
<td>2.5 (0.7-4.1)</td>
<td>3.4 (2.1-4.3)</td>
<td>1.7 (0.7-2.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-transfused</td>
<td>1.4 (0.6-2.9)</td>
<td>2.1 (0.6-3.2)</td>
<td>2.2 (1.0-5.4)</td>
<td>1.9 (1.1-2.6)</td>
<td>1.3 (0.7-2.0)</td>
<td>2.0 (0.7-4.7)</td>
</tr>
<tr>
<td>CD5(+) T cells</td>
<td>67 (37-86)</td>
<td>Transfused</td>
<td>75 (53-83)</td>
<td>52 (21-83)</td>
<td>62 (32-88)</td>
<td>54 (33-89)</td>
<td>72 (56-87)</td>
<td>65 (44-76)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-transfused</td>
<td>63 (27-86)</td>
<td>65 (35-80)</td>
<td>67 (49-81)</td>
<td>62 (29-90)</td>
<td>65 (43-77)</td>
<td>55 (48-72)</td>
</tr>
<tr>
<td>CD2(+) T cells</td>
<td>83 (61-90)</td>
<td>Transfused</td>
<td>77 (51-95)</td>
<td>70 (39-90)</td>
<td>80 (46-95)</td>
<td>59 (35-91)</td>
<td>78 (62-88)</td>
<td>86 (73-93)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-transfused</td>
<td>85 (60-95)</td>
<td>76 (48-94)</td>
<td>83 (62-95)</td>
<td>86 (77-94)</td>
<td>87 (52-94)</td>
<td>83 (52-94)</td>
</tr>
<tr>
<td>CD19(+) B cells</td>
<td>13 (1-53)</td>
<td>Transfused</td>
<td>4.0 (1.8-7.0)</td>
<td>5.5 (3.3-8.8)</td>
<td>7.7 (3.4-13)</td>
<td>11.3 (7.1-14)</td>
<td>4.9 (4.1-6.6)</td>
<td>9.7 (5.9-14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-transfused</td>
<td>6.2 (1.5-11)</td>
<td>8.5 (2.8-24)</td>
<td>9.2 (2.3-16)</td>
<td>5.6 (0.7-8.5)</td>
<td>5.6 (2.9-7.9)</td>
<td>7.8 (3.9-12)</td>
</tr>
</tbody>
</table>

a p = <0.05 versus baseline value on day of surgery.
b p = 0.052 versus corresponding value in transfused patients
transfused and non-transfused patients, there were no significant changes for CD3(+) T cells, CD2(+) T cells, or CD19(+) B cells during the post-operative courses. Compared to baseline values on the day of operation, an increase of CD4 T cells occurred on da 7 and a decrease of CD8 T cells occurred on da 4 in the non-transfused patients. CD4/CD8 ratio was increased on da 4 and 30 post-gastrectomy in the transfused group and on da 4 and 7 in the non-transfused group.

As shown in Table 3, transfused patients showed a slight (statistically insignificant) increase in Th1/Th2 ratio at 4 da post-gastrectomy. There were no significant differences in Th1/Th2 ratio between transfused and non-transfused patients on any of the specified days post-gastrectomy. There were also no significant differences between the 2 groups in regard to total hospital charges or duration of antibiotic use. The only significant difference was a longer hospital stay for transfused patients (mean 20.5 da) compared to non-transfused patients (mean 16.2 da).

Discussion

In this study, peripheral blood lymphocyte subsets were measured by flow cytometry in gastric cancer patients after gastrectomy, with or without perioperative blood transfusion(s). No significant differences between the treatment groups were found in respect to CD3(+) T cells, CD2(+) T cells, or CD19(+) cells. Slight decrease of CD8 T cells on the 4th postoperative da, slight increase of CD4 T cells on the 7th da, and increased CD4/CD8 ratios on the 4th and 30th da occurred in the transfused patients. Similar increases in CD4/CD8 ratios occurred on da 4 and 7 in non-transfused patients. No significant differences were observed for CD4 T cells, CD8 T cells, or CD4/CD8 ratios between the transfused and non-transfused groups.

Beneficial and detrimental effects of transfusion immunomodulation have both been recognized in the surgical setting [3]. Surgery, per se, evidently contributes to a deviation of the immune response toward a Th2 pattern [36], and the degree of immunomodulatory effect is probably proportional to surgical trauma [18,37]. Thus, we consider the fluctuations that were observed in CD4 T cells, CD8 T cells, and the CD4/CD8 ratio to be associated with surgical trauma or related morbidities.

In this study, the technology of intracytoplasmic cytokine detection was used to determine the ratio of Th1/Th2 cells in gastric cancer patients after gastrectomy, with or without blood transfusion. An increase in Th1/Th2 ratio occurred in transfused patients on the 4th postoperative day, compared to the baseline values (Table 3).

There were no significant differences between the transfused or non-transfused patients in Th1/Th2 ratio, total hospital charges, or duration of antibiotic utilization. These findings are contrary to expectations, since these variables have all been suggested to reflect the immunomodulatory effects of blood transfusion [1-4,13,19-24].

There are many confounding factors, comorbid conditions, and parameters (eg, surgical procedure, age, gender, clinical stage of disease, perioperative infection) that may influence immunomodulatory effects [16,18,20,36-37]. The number of patients in this study was too small for a meaningful multivariate analysis. The only variable that showed a significant difference between the transfused and non-transfused groups was the modest increase in the duration of hospital stay that was observed in the transfused patients.

Jensen et al [12] showed in a randomized study that recipients of leukocyte-depleted allogeneic transfusions had a substantially lower incidence of post-operative morbidity, compared to the recipients of unmodified allogeneic transfusions (2% versus 23%, respectively). This suggests that unmodified allogeneic transfusions can exert an influence on host defenses against infection that may be mediated by transfused leukocytes.

Administration of leukocyte-depleted blood to patients who need perioperative transfusion may help to avoid untoward effects of allogeneic transfusion [13]. In a future study, in addition to including many more patients, it will be warranted to include one transfused group that receives unmodified allogeneic blood and another transfused group that receives leukocyte-depleted blood.

Acknowledgements

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References


