Feline CD8+ T cell non-cytolytic anti-feline immunodeficiency virus activity mediated by a soluble factor(s)

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Feline immunodeficiency virus (FIV) is more readily isolated from CD8+ T cell-depleted peripheral blood mononuclear cells (PBMC) of FIV-infected cats than from unFractioned PBMC cultures. However, it is not known whether feline CD8+ T cells down-regulate FIV expression by direct interaction with FIV-infected cells or via a soluble mediator. Furthermore, it is not known whether this anti-FIV activity involves a lytic or non-lytic mechanism. In the present study, we demonstrated that autologous and allogeneic CD8+ T cells from asymptomatic FIV-infected cats inhibited the replication of FIV in CD8+ T cell-depleted PBMC cultures in a dose-dependent manner. The inhibitory effect was mediated by a non-lytic mechanism, and was not dependent on direct cell-to-cell contact: an inhibitory effect was exerted by CD8+ T cells across a semi-permeable membrane, and an inhibitory activity was also present in cell-free supernatants from CD8+ T cells. These results suggest that this suppressive effect is mediated, at least in part, by soluble factors produced by CD8+ T cells.

Introduction

It is well known that human immunodeficiency virus (HIV) infection induces various immune responses in humans. Cytokines, including interferon, TNF, interleukin 16 (IL-16), MIP-1α, MIP-1β and RANTES, as well as anti-HIV antibody production and the induction of cellular immunity are involved in HIV replication, and the balance between these factors controls HIV growth (Biswa et al., 1992; Poli & Fauci, 1992, 1993; Montaner et al., 1993; Weissman et al., 1994; Fauci, 1996). During the early acute stage of HIV infection, bursts of virus replication and high levels of plasma viraemia are observed, and then the infection enters the long clinically asymptomatic stage (Fauci, 1993). It is considered that the induction of HIV-specific CD8+ cytotoxic T lymphocytes (CTL) is important in maintaining this asymptomatic stage (Walker & Plata, 1990). These CTL are major histocompatibility complex (MHC) class I molecule-restricted and destroy HIV-infected cells, recognizing epitopes of Gag, reverse transcriptase (RT), Nef or envelope (Env) proteins (Levy et al., 1996). HIV growth is detected with relative ease in PBMC obtained from patients with active AIDS in a medium containing IL-2. However, although HIV can be isolated by co-cultivating PBMC from asymptomatic HIV patients with PBMC from uninfected donors, virus growth is hardly detected when PBMC from asymptomatic patients are cultured alone. A high level of HIV growth can be detected with these PBMC in the asymptomatic stage when CD8+ T cells are eliminated from the PBMC. This inhibitory effect of CD8+ T cells on HIV replication was initially reported by Walker et al. (1986). This inhibition of HIV replication is non-cytotoxic and there is no decrease in the number of HIV-infected cells (Walker et al., 1986, 1991a). Anti-HIV activity was demonstrated with a trans-well culture device in which CD8+ T cells were separated from infected CD4+ T cells by a semi-permeable membrane, and also by exposing infected CD4+ T cells to filtered supernatants from cultured CD8+ cells (Walker & Levy, 1989; Brinchmann et al., 1990). Further, this inhibition can be induced by MHC-mismatched CD8+ T cells (Brinchmann et al., 1990; Mackeweicz et al., 1991; Walker et al., 1991b). Therefore, it is clear that the anti-HIV activity of CD8+ T cells is not due to CTL activity, but to a soluble factor(s) produced by these CD8+ T cells. Thus, the non-MHC-restricted non-cytotoxic anti-HIV response of CD8+ T cells together with MHC-restricted CTL is important in maintenance of the asymptomatic stage of HIV infection.

Feline immunodeficiency virus (FIV) was originally isolated in 1986 from a feline leukaemia virus-negative cat with chronic...
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otic infections (Pedersen et al., 1987). FIV is a typical lentivirus with its ultrastructural morphology, structural protein profile and reverse transcriptase requirement resembling those of HIV. Similar to HIV infection, plasma viraemia is detected during the early stage of FIV infection. Thereafter, a long clinically asymptomatic stage ensues, during which the number of CD4+ T cells gradually decreases, suggesting eventual induction of the clinical signs of AIDS (Yamamoto et al., 1988; English et al., 1994; Kohimoto et al., 1998). Jeng et al. (1996) reported that FIV is more readily isolated from CD8+ T cell-depleted PBMC of FIV-infected cats than from unfraccionated PBMC cultures, as in HIV infections, although detection of infectious virus in the plasma is difficult during this asymptomatic stage. However, their report did not describe whether this anti-FIV activity of CD8+ T cells was mediated by soluble factors produced by CD8+ T cells.

Therefore, in this study, we investigated whether CD8+ T cell anti-FIV activity requires cell-to-cell contact or if activation occurs via non-cytotoxic soluble factors.

Methods

**Cell culture.** PBMC, CD8+ T cell-depleted PBMC and CD8+ T cells were cultured in RPMI 1640 growth medium supplemented with 10% heat-inactivated foetal calf serum (FCS), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), 50 μM 2-mercaptoethanol, 2 μg/ml polybrene, 100 U/ml recombinant human IL-2 and 10 μg/ml concanavalin A (Con A). The medium was replaced every 3 days with fresh medium without Con A.

**Experimental animals.** Eight experimentally FIV-infected cats and one naturally infected cat were used in this study. The experimentally infected cats, KF610, KF621, KF753, KF769, KF863, KF879, KF880 and KF883, were intraperitoneally injected with 1 ml blood obtained from one of eight naturally FIV-infected cats of different origins. The cats remained in the asymptomatic stage of the disease for the length of the study, and remain clinically healthy after the following periods: KF610, 230 days; KF621, 820 days; KF753, 1620 days; KF769, 1620 days; KF863, 580 days; KF879, 570 days; KF880, 980 days; and KF883, 450 days. KF876, a naturally FIV-infected cat, has also remained clinically healthy for at least 1000 days since we started keeping it in our laboratory.

**Antibodies.** Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (MAb) against feline CD4 (fCD4), phycoerythrin (PE)-conjugated MAb against feline CD5 (fCD5) and FITC-conjugated MAb against feline CD8 (fCD8) were used for detecting CD4, CD5 (Pan T) and CD8 antigens, respectively, by flow cytometry. These antibodies were purchased from Southern Biotechnology Associates.

For depletion and separation of CD8+ T cells, a MAb against human CD8+ T cells, OKT8 (ATCC CRL8014), was used. MAb OKT8 recognizes the α-chain of human CD8 antigen and cross-reacts with feline CD8+ T cells.

**Complement.** Blood collected from rabbit heart was incubated for 1 h at room temperature, then 1 h at 4 °C. The blood was then centrifuged at 2500 r.p.m. at 4 °C for 10 min, and the rabbit serum obtained was used as complement. Complement activity was confirmed by haemolytic assays.

**Flow cytometric analysis.** A total of 1 x 106 cells was incubated with FITC-conjugated fCD4 MAb and PE-conjugated f43 MAb, or FITC-conjugated fCD8 MAb and PE-conjugated f43 MAb, at 4 °C for 1 h. The cells were washed three times, and the number of stained cells was determined by counting about 10000 cells on a fluorescence-activated cell sorter (FACS 440, Becton Dickinson). Absolute lymphocyte numbers were determined from complete blood count and differential cell count.

**Depletion of CD8 T cells from PBMC.** PBMC (1 x 107 cells) were incubated with 500 μl purified MAb OKT8 IgG at 4 °C for 30 min, and then reacted with 8 U complement in the same volume for 1 h at 37 °C. The cells were then washed three times with Hanks’ balanced salt solution (HBSS), and used as CD8+ T cell-depleted PBMC in the experiments.

**Separation of CD8 T cells from PBMC.** PBMC (1 x 107 cells) were incubated with 500 μl purified MAb OKT8 IgG at 4 °C for 30 min. The cells were then washed three times with PBS containing 2 mM EDTA and 0.5% BSA, and then 40 μl micro-beads coated with rat anti-mouse IgG2a + b (Miltenyi Biotec) was added to the cells and the mixture was incubated at 6 °C for 15 min. After washing with PBS containing 2 mM EDTA and 0.5% BSA, the cells were fractionated into CD8+ T cells and CD8- T cell-depleted cells by the magnetic system using Mini MACS (Miltenyi Biotec).

**p24 FIV antigen assay.** p24 FIV antigen was measured with a commercial ELISA kit (IDEXX).

Results

FIV replication in unseparated and CD8+ T cell-depleted PBMC from cats infected with FIV

PBMC collected from eight experimentally infected cats and one naturally infected cat were reacted with MAb OKT8 and rabbit complement to eliminate CD8+ T cells. The elimination was confirmed to be limited to CD8+ T cells by detecting the cell population by flow cytometry using MAb fCD8, which recognizes an epitope differing from that recognized by OKT8 (Fig. 1A, B). These CD8+ T cell-depleted PBMC and unseparated PBMC were cultured and the amounts of FIV p24 antigen produced in the culture fluids were compared to investigate the anti-FIV activity of CD8+ T cells. As shown in Table 1, although FIV p24 antigen was detected in the culture fluids of both unseparated PBMC and CD8+ T cell-depleted PBMC collected from two cats, KF621 and KF769, FIV p24 antigen was detected only in the CD8+ T cell-depleted PBMC culture fluid in other cats. The CD4/CD8 cell ratios of KF621 and KF769, which did not show any difference in the elevation of FIV antigen in the culture fluids between unseparated PBMC and CD8+ T cell-depleted PBMC, were 0.5 or lower.

Effect on FIV replication of reconstituting CD8+ T cell-depleted PBMC with autologous CD8+ T cells

CD8+ T cell-depleted PBMC collected from KF863 and KF879 were cultured with 1 x 106 or 1 x 105 autologous CD8+ T cells, and the effect on FIV replication was inves-
CD8\(^+\) T cell anti-FIV activity

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![Flow cytometric analysis of cells double-stained with FITC-conjugated MAb fCD8 (CD8) and PE-conjugated MAb f43 (Pan T).](image)

Fig. 1. Flow cytometric analysis of cells double-stained with FITC-conjugated MAb fCD8 (CD8) and PE-conjugated MAb f43 (Pan T). (A) intact PBMC; (B) PBMC treated with MAb OKT8 and rabbit complement; (C) cell population isolated by the magnetic system; and (D) cell population depleted of CD8\(^+\) T cells by the magnetic system.

As shown in Fig. 2, FIV p24 antigen levels in the CD8\(^+\) T cell-depleted PBMC culture started to increase after 15–18 days of culture, showing FIV replication. However, when CD8\(^+\) T cell-depleted PBMC were cultured with 1 \times 10^6 autologous CD8\(^+\) T cells, FIV replication was inhibited, as it was in the unseparated PBMC culture. This inhibition was dependent on the number of the CD8\(^+\) T cells added, as the effect was weaker when 1 \times 10^4 CD8\(^+\) T cells were added.

**Anti-FIV activity of allogeneic CD8\(^+\) T cells**

CD8\(^+\) T cells from KF863 and KF879 were added to CD8\(^+\) T cell-depleted PBMC from KF879 and KF863, respectively, to prepare allogeneic cell mixtures, and the anti-FIV activity of allogeneic CD8\(^+\) T cells was investigated. As shown in Fig. 3, FIV p24 antigen was not detected in cultures of unseparated PBMC from KF863 or KF879, while it started to increase after 15–18 days of culture in CD8\(^+\) T cell-depleted PBMC cultures. When these CD8\(^+\) T cell-depleted PBMC were cultured with autologous CD8\(^+\) T cells, FIV replication was clearly inhibited. Further, this anti-FIV activity was inhibited when allogeneic CD8\(^+\) T cells were added, although the inhibitory effect was weaker than that of autologous CD8\(^+\) T cells.

**Relationship between CD8\(^+\) T cell anti-FIV activity and cellular cytotoxicity**

PBMC from KF883 were separated into two groups, CD8\(^+\) T cell-depleted PBMC and unseparated PBMC, and cultured at 37 °C. After 15 days of culture, the unseparated PBMC were divided into two groups, CD8\(^+\) T cells were eliminated from

<table>
<thead>
<tr>
<th>Cat</th>
<th>CD4/CD8 cell ratio</th>
<th>Maximum FIV p24 antigen in culture supernatants*</th>
<th>Maximum FIV p24 antigen (day)†</th>
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<tr>
<td></td>
<td>Unseparated PBMC</td>
<td>CD8(^+) T cell-depleted PBMC†</td>
<td></td>
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<tr>
<td>KF610</td>
<td>1:30</td>
<td>0.05 ± 0.02</td>
<td>1.43 ± 0.21</td>
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<tr>
<td>KF621</td>
<td>0.35</td>
<td>1.01 ± 0.16</td>
<td>1.16 ± 0.11</td>
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<tr>
<td>KF753</td>
<td>1:92</td>
<td>0.06 ± 0.01</td>
<td>1.05 ± 0.07</td>
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<tr>
<td>KF769</td>
<td>0.49</td>
<td>0.05 ± 0.01</td>
<td>0.96 ± 0.12</td>
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<tr>
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<td>0.05 ± 0.01</td>
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<td>KF876</td>
<td>0.78</td>
<td>0.05 ± 0.02</td>
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<td>KF879</td>
<td>0.85</td>
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<tr>
<td>KF880</td>
<td>0.45</td>
<td>0.06 ± 0.01</td>
<td>1.29 ± 0.15</td>
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<tr>
<td>KF883</td>
<td>0.51</td>
<td>0.05 ± 0.03</td>
<td>1.22 ± 0.18</td>
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</table>

* The cell concentration was adjusted to 10^6 cells/ml, and FIV p24 antigen in the supernatants was measured every 3 days before replacement of culture medium. Results are expressed as O.D. at 595 nm, mean ± SD.
† PBMC were treated with OKT8 and complement to deplete CD8\(^+\) lymphocytes.
‡ Day of culture on which maximum levels of FIV p24 antigen were detected.
Fig. 2. Effect on FIV replication of reconstituting CD8$^+$ T cell-depleted PBMC cultures with autologous CD8$^+$ T cells. CD8$^+$ T cell-depleted PBMC obtained from cats KF863 (A) and KF879 (B) were cultured with autologous CD8$^+$ T cells, and the amounts of FIV antigen in the culture fluid were compared. CD8$^+$ T cell-depleted PBMC (1 x 10^6 cells) were cultured alone (○) or with 1 x 10^6 (▲) or 1 x 10^5 (■) autologous CD8$^+$ T cells added prior to the initiation of culture. A control culture of 1 x 10^6 unseparated PBMC (●) was also established. All culture supernatants were monitored for FIV p24 antigen at 3 day intervals.

one group, and all cells were cultured for a further 15 days. As shown in Fig. 4, FIV replication was not detected at all in the unseparated PBMC that had been cultured for 30 days, while an immediate increase in FIV p24 antigen was observed in the PBMC culture from which CD8$^+$ T cells were depleted at the beginning of culture. Further, when CD8$^+$ T cells were depleted from the unseparated PBMC on the 15th day of culture, FIV p24 antigen increased immediately thereafter. These results suggest that the inhibition of FIV replication was not caused by direct injury of FIV-infected cells by CD8$^+$ T cells.

Effect of culturing CD8$^+$ T cells separated from FIV-infected PBMC by a semi-permeable membrane

CD8$^+$ T cell-depleted PBMC from KF876 were cultured with autologous CD8$^+$ T cells with a membrane filter (0.45 µm) separating the two cell populations to determine whether the anti-FIV activity of CD8$^+$ T cells was mediated by soluble factors. As shown in Fig. 5, although the CD8$^+$ T cells were separated by a membrane filter, FIV replication was inhibited, as was observed when CD8$^+$ T cell-depleted PBMC were mixed with CD8$^+$ T cells. However, the inhibitory effect was less than that in the mixed culture of CD8$^+$ T cell-depleted PBMC and CD8$^+$ T cells.

Anti-FIV activity of cell-free supernatants from CD8$^+$ T cells

The supernatant of a 9–15 day culture of CD8$^+$ T cells from KF876 was passed through a 0.45 µm filter, and a medium containing 30% filtrate was prepared. A medium containing 30% culture supernatant of CD8$^+$ T cells obtained from an
However, when CD8+ cells from an FIV-negative cat and with the usual medium. CD8+ medium containing the culture supernatant of autologous PBMC (Fig. 4). CD8+ PBMC (1 x 10^6 cells) were divided into two groups, CD8+ cell-depleted PBMC (■) and unseparated PBMC (○), and then cultured at 37 °C. After 15 days of growth, the unseparated PBMC were divided again into two groups, and CD8+ T cells were depleted from one group (●), and then cultured further. Culture supernatants were monitored for FIV p24 antigen at 3 day intervals.

In seven cats (all except KF621 and KF763), FIV replication was observed only in CD8+ T cell-depleted PBMC. When these CD8+ T cell-depleted PBMC were reconstituted with allogeneic CD8+ T cells, FIV replication was inhibited in a manner dependent on the number of added CD8+ T cells. A reconstitution study with CD8+ T cells was not performed in the report of Jeng et al. (1996), so this is the first study to report on the FIV system. The result of this reconstitution study confirms that the CD8+ T cells of FIV-infected cats have an antiviral activity similar to that observed in HIV infection. Since this antiviral activity was effective when CD8+ T cell-depleted PBMC were cultured with allogeneic CD8+ T cells, the activity is non-MHC-restricted, the same as that in the HIV system. Further, since FIV was immediately released when CD8+ T cells were depleted from non-virus producing PBMC, the activity is non-MHC-restricted, the same as that in the HIV system. Since FIV was immediately released when CD8+ T cells were depleted from non-virus producing PBMC, the activity is non-MHC-restricted, the same as that in the HIV system.

Discussion

Jeng et al. (1996) demonstrated that FIV is more readily isolated from CD8+ T cell-depleted PBMC of FIV-infected cats than from unfractionated PBMC cultures. However, it is not known whether feline CD8+ T cells down-regulate FIV expression by direct interaction with FIV-infected cells or via a soluble mediator. Furthermore, it is not known whether anti-FIV activity involves a lytic or nonlytic mechanism. In this study, we investigated the anti-FIV activity of CD8+ T cells from eight experimentally and one naturally FIV-infected cats. In seven cats (all except KF621 and KF763), FIV replication was observed only in CD8+ T cell-depleted PBMC. When these CD8+ T cell-depleted PBMC were reconstituted with allogeneic CD8+ T cells, FIV replication was inhibited in a manner dependent on the number of added CD8+ T cells. A reconstitution study with CD8+ T cells was not performed in the report of Jeng et al. (1996), so this is the first study to report on the FIV system. The result of this reconstitution study confirms that the CD8+ T cells of FIV-infected cats have an antiviral activity similar to that observed in HIV infection. Since this antiviral activity was effective when CD8+ T cell-depleted PBMC were cultured with allogeneic CD8+ T cells, the activity is non-MHC-restricted, the same as that in the HIV system. Further, since FIV was immediately released when CD8+ T cells were depleted from non-virus producing PBMC cultured for 15 days, it is suggested that this anti-FIV activity was not cytotoxic, differing from the CTL activity.

CD8+ T cells were not able to pass through the 0.45 μm membrane filter used to separate CD8+ T cell-depleted PBMC and autologous CD8+ T cells in co-culture experiments; therefore the anti-FIV activity must be mediated by soluble factors. This was confirmed by the fact that anti-FIV activity...
was also mediated by culture medium containing the cell-free supernatant of a CD8⁺ T-cell culture. However, more effective anti-FIV activity was induced when CD8⁺ T-cell-depleted PBMC were cultured directly with CD8⁺ T cells. This suggests that production and/or delivery of anti-FIV activity to target cells is more efficient when cells are in close contact. Although the sources of anti-FIV activity are not clear, that induced by soluble factors and that induced by cell-to-cell contact may be mediated by different mechanisms. The observation that more effective anti-FIV activity was induced by autologous CD8⁺ T cells than by allogeneic CD8⁺ T cells might also be related to the fact that more effective anti-FIV activity was induced by cell-to-cell contact. In any case, it is clear that the suppressive effect of CD8⁺ T cells is at least partly mediated by soluble factors produced by those cells.

A major effort has been directed towards the identification and characterization of the anti-HIV ‘lymphokine’ produced by CD8⁺ T cells. Anti-HIV ‘lymphokines’ have been identified using many recombinant human cytokines and monoclonal antibodies against these cytokines. However, the anti-HIV active substance from CD8⁺ T cells has not yet been identified (Mackewicz & Levy, 1992; Mackewicz et al., 1994; Greenberg et al., 1997). Recently, it was reported that β-chemokines (RANTES, MIP-1α and MIP-1β) produced by CD8⁺ T cells inhibit HIV replication (Cocchi et al., 1995). Further, it was reported that receptors for chemokines including PBL/SDF-1, RANTES, MIP-1α and MIP-1β function as the second receptors (co-receptors) for HIV (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996). However, it was reported that CD8⁺ T cell anti-HIV activity was not completely inhibited by antibodies against β-chemokines, suggesting that β-chemokines are not the only antiviral factors produced by CD8⁺ T cells (Moriuchi et al., 1996; Rubbert et al., 1997). Although the identity of the primary high-affinity receptor for FIV remains elusive, it was recently reported that FIV that has been selected for growth in a Crandell feline kidney cell line (CrFK-tropic virus) is able to use CXCR4, which is an α-chemokine receptor, for cell fusion, and that human SDF-1 inhibits CrFK-tropic FIV infection of CrFK cells (Hosie et al., 1998; Willett et al., 1997). In contrast, when an IL-2-dependent feline T-cell line (Mya-1) was used instead of CrFK cells, the inhibitory activity of SDF-1 was not detected, suggesting that a CXCR4-independent mechanism of infection is also present (Hosie et al., 1998). In any case, our results in this study show that factors produced by CD8⁺ T cells are also effective on cells containing integrated FIV proviral DNA. That is, anti-FIV activity affected the cells after FIV proviral DNA was integrated into the cellular DNA, undermining the suggestion that virus replication is inhibited by blocking absorption of the virus by chemokines. It has been reported that inhibition of HIV replication by the CD8⁺ T cell antiviral activity is not achieved through blocking of the viral absorption step, but through blocking of HIV mRNA transcription (Chen et al., 1993; Mackewicz et al., 1995). It was reported recently that IL-16 secreted by CD8⁺ T cells inhibits HIV mRNA synthesis (Baier et al., 1995; Zhou et al., 1997), suggesting that IL-16 is a CD8⁺ T cell antiviral factor.

It is reported that in HIV infection, CD8⁺ T cell anti-HIV activity is correlated with clinical stage until the onset of AIDS (Mackewicz et al., 1991; Landay et al., 1993; Gomez et al., 1994). All cats used in this study were at the asymptomatic stage of FIV infection, and CD4/CD8 ratios were distributed within the range 0.35–1.92; the ratio was above 1:0 in two cats, 0.5 to 1:0 in three cats and below 0:5 in four cats. The CD4/CD8 ratios of the two cats that did not show CD8⁺ T cell anti-FIV activity were as low as 0.35 and 0.49. The relation of CD8⁺ T cell anti-FIV activity to clinical stage until the onset of AIDS and to CD4/CD8 cell ratio needs to be investigated in detail.

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References


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