Vitamin K2 ameliorates experimental autoimmune encephalomyelitis in Lewis rats

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Abstract

Vitamin K2 (VK2), which has been in wide use for the management of hypoprothrombinemia and osteoporosis in Japan, was tested for its efficacy on experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). The severity of EAE was significantly ameliorated by the prophylactic administration of VK2, though it was not effective when given after the onset. Inflammatory cellular infiltration and the expression of both MHC class II and inducible nitric oxide synthase (iNOS) were reduced in the spinal cords of VK2-treated rats with EAE. The inhibitory effect of VK2 on the iNOS expression in glial cells was also observed in vitro. Considering the long use of VK2 without noticeable untoward effects, it may be applicable to the patients with MS.

Keywords: Vitamin K2; Experimental autoimmune encephalomyelitis; MHC class II; Inducible nitric oxide synthase

1. Introduction

Experimental autoimmune encephalomyelitis (EAE), an inflammatory demyelinating disease of the central nervous system (CNS) provoked by myelin antigens, is widely used as an animal model of multiple sclerosis (MS) (Zamvil and Steinman, 1990). Although there are drugs available for MS such as corticosteroids, interferon-β, glatiramer acetate, and immunosuppressants, the effects of these drugs are not sufficient for the management of MS.

Vitamin K2 (VK2, menatetrenone, menaquinone-4) is a cofactor of γ-glutamyl-carboxylase, which converts glutamic acid into γ-carboxyglutamic acid, and is essential for γ-carboxylation of certain proteins such as prothrombin (Stenflo et al., 1974). However, several functions of VK2 independent of γ-carboxylation have been reported. VK2 has an inhibitory effect on osteoporosis by inhibiting bone loss induced by treatment with corticosteroids, ovariectomy, and sciatic neurectomy (Akiyama et al., 1993; Hara et al., 1993a; Iwasaki-Ishizuka et al., 2003). Though the mechanisms how VK2 inhibits bone loss are not clearly understood, the inhibitory effects on the expression of cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), and some inflammatory cytokines have been suggested to be involved (Akiyama et al., 1994; Hara et al., 1993b; Hiruma et al., 2004). Tumor cell invasion is inhibited by VK2 by activating protein kinase A, which leads to the inhibition of RhoA activity (Otsuka et al., 2004). VK2 inhibits tendency to arterial thrombosis in a rat model, and its side chain is suggested to have an important role in this effect (Ronden et al., 1997). The effects of VK2 on neural cells are also reported. Cell death of oligodendrocyte precursors and immature neurons induced by oxidative injury is protected by VK2, and the inhibitory effect on the production of reactive oxygen species (ROS) has been suggested (Li et al., 2003). Thus, VK2 has a variety of effects such as inhibition of the synthesis of inflammation-related molecules and...
3. Results

3.1. ROS, and activating protein kinase A, which are supposed to have the beneficial effect on EAE.

The inhibition of geranylgeranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate (FPP) synthesis plays a key role in the effects of 3-hydroxy-3-methylglutal-coenzyme A (HMG-CoA) reductase inhibitors (statins), and it was demonstrated by many researchers that statins have beneficial effects on EAE (Aktas et al., 2003; Greenwood et al., 2003; Stanislaus et al., 2001; Youssef et al., 2002). Since the side chain of VK2, geranylgeraniol (GGO), has common structure to geranylgeranyl-pyrophosphate (GGPP), it could be expected that the side chain of VK2 had similar effects as seen in statins.

These facts prompted us to investigate the effects of VK2 on EAE and we report here the efficacy of VK2 on the severity of EAE and discuss the mechanisms underlying.

2. Materials and methods

2.1. Induction, treatment, and clinical evaluation of actively induced EAE

Female Lewis rats at 8 weeks old were obtained from Japan SLC (Hamamatsu, Japan). Induction of EAE was performed as previously described (Fujimoto et al., 1999). Briefly, rats were immunized in both hind footpads with 100 μl of encephalitogenic mixture containing 100 μg bovine myelin basic protein (MBP, Sigma, St Louis, MO, USA) in complete Freund’s adjuvant (Difco, Detroit, MI, USA) supplemented with 200 μg of H37Ra Mycobacterium tuberculosis (Difco).

A hundred and five rats were subjected to the analyses in total. For a high-dose prophylactic trial, 62.5 mg/kg of VK2 (Kobayashi Kakou, Fukui, Japan) was intraperitoneally administered daily, from one day before immunization (day −1) to twenty-three days after immunization (day 23). Control rats received the same amount of vehicle consisting of polyoxyethylene caster oil 60 (60 mg/ml, Nihon Surfactant Kogyo, Tokyo, Japan), sesquioleate sorbitan (10 mg/ml, Nihon Surfactant Kogyo), polysorbate 80 (20 mg/ml, Nihon Surfactant Kogyo), and propylene glycol (30 mg/ml, Wako Pure Chemical Industries, Osaka, Japan) solved in distilled deionized water. Rats were subjected to the evaluation of the clinical scores (VK2-treated group, n = 18; vehicle-treated group, n = 17), or histological/immunochemical analysis (VK2, n = 6; vehicle, n = 6). For a low-dose prophylactic trial, 2.7 mg/kg of VK2 or vehicle was administered intraperitoneally from day −1 to 23 (VK2, n = 6; vehicle, n = 6). For a therapeutic trial, 62.5 mg/kg of VK2 or vehicle was administered intraperitoneally from day 11 to 19 (VK2, n = 18; vehicle, n = 17).

The severity of EAE was evaluated in a blind fashion as we previously described: 0, normal; 1, limp tail; 2, mild paraparesis of the hind limbs with unsteady gait; 3, moderate paraparesis with preservation of voluntary movement; 4, paraplegia (Fujimoto et al., 1999). Experimental procedures were approved by the institutional Animal Care and Use Committee of Osaka University Graduate School of Medicine.

2.2. Histology and immunohistochemistry

Histological and immunohistochemical analysis were performed for a prophylactic trial. Twelve rats were immunized, and half of them were treated with VK2 and the remaining were treated with vehicle, then three rats of each group were sacrificed on days 13 and 15 for evaluation in a blinded fashion. Rats were anesthetized with isoﬂurane and perfused transcardially with ice-cold physiological saline, and lumbar spinal cords were removed and immersed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C overnight. The lower halves of the tissues were embedded in paraffin for histological analysis. The upper halves of the tissues were immersed in 30% sucrose in PBS at 4 °C overnight, embedded in OCT compound, and frozen in liquid nitrogen for immunohistochemistry. Five paraffin sections of 4 μm thick were cut at 0.1 mm intervals from each rat and stained with hematoxylin–eosin. Semi-quantitative histological analysis of inflammatory cellular infiltration was performed using the following score: 0, no inflammation; 1, cellular infiltrates only in the perivascular areas and meninges; 2, mild cellular infiltration in parenchyma; 3, moderate cellular infiltrates in parenchyma; 4, severe cellular infiltrates in parenchyma (Okuda et al., 2002). For immunohistochemical analysis, cryosections (10 μm thick) were incubated with mouse anti-I-A antibody (1:150, Immunotech, Marseille, France) or rabbit anti-iNOS antibody (1:50, BD Biosciences Pharmingen, San Diego, CA, USA) in PBS containing 0.1% bovine serum albumin at 4 °C overnight. Sections were then incubated with biotin-conjugated anti-mouse IgG antibody (1:16,000, Vector Laboratories, Burlingame, CA, USA) or biotin-conjugated anti-rabbit IgG antibody (1:1800, Vector Laboratories) for 2 h and incubated with streptavidin-conjugated Texas red (1:400, Molecular Probes, Eugene, OR, USA) for 30 min. For glial cell labeling, sections were incubated with microglial marker FITC-conjugated tomato-lectin (1:200, Sigma), or astrocyte marker rabbit anti-GFAP (1:400, DAKO, Denmark) or mouse anti-GFAP (1:500, Sigma) antibodies, followed by appropriate secondary antibodies.

2.3. Cell culture

Microglial cell line GMI-M6-3 (Kanzawa et al., 2000) were grown in Minimum Essential Medium (Sigma) containing 10% fetal bovine serum (FBS), 0.2% glucose, and 5 μg/ml of bovine insulin (Sigma). Astrocytes were prepared as described previously (Koguchi et al., 2002). Cells collected from cerebral cortices from neonatal mice were maintained in media consisting of Dulbecco’s modi-
fied Eagle’s medium (DMEM, Nikken Bio Medical Laboratory, Kyoto, Japan) containing 10% FBS. Cells were incubated with 25 μM of VK2 or vehicle for 24 h, followed by the incubation with recombinant mouse interferon-γ (Genzyme-Techne, Cambridge, MA, USA), agonistic anti-CD40 antibody (HM40-3, BD Bioscience Pharmingen), lipopolysaccharide (LPS, Sigma), or the combination of these chemicals for further 24 h. Then cells were collected and their protein extracts were subjected to Western blot analysis. For cellular toxicity assay, GMI-M6-3 cells were incubated in 200 μl of medium containing VK2 (25 or 100 μM) or vehicle in a 96-well microtiter plate. A 20 μl of Cell Counting kit-8 (WST-8, Dojindo, Kumamoto, Japan) was added to each well after 44 h, followed by 4 h incubation, then optical density was measured at 450 nm using Emax (Molecular Devices).

2.4. Western blot analysis

Western blot analysis was carried out as previously described (Okuno et al., 2004). Samples were lysed with RIPA buffer [PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), PH7.4] containing protease inhibitors (20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1 μg/ml leupeptin). The same amount of total protein extracts were resolved on 10% SDS–polyacrylamide gels, and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The blots were incubated with mouse anti-I-A antibody (1 :150), rabbit anti-iNOS antibody (1 :150), rabbit anti-β-actin (1 :20,000, Sigma) at 4 °C overnight. The blots were subsequently incubated with an appropriate secondary antibodies conjugated with horseradish peroxidase for 45 min and visualized by enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotechnology, Piscataway, NJ). The image of the film was captured and analyzed using Fluorchem IS8000 (Alpha Innotech, San Leandro, CA, USA), which allows quantification of intensity of the bands. The relative intensity of each band was determined and normalized to the intensity of β-actin.

2.5. Statistical analysis

Statistical analysis was performed with Kolmogorov–Smirnov test for disease duration, and with Student’s t-test.
for clinical score, histological score, and semi-quantitative Western blot analysis.

3. Results

3.1. Treatment with VK2 ameliorates the severity of EAE in a prophylactic manner

The intraperitoneal administration of VK2 (62.5 mg/kg) from day –1 to 23 significantly reduced the mean clinical score at days 15, 16, and 17 of actively induced EAE (Fig. 1A). Treatment with VK2 in a prophylactic use shortened the disease duration (Fig. 1B), and reduced the integral of the area under the curve (AUC) of the clinical score (Fig. 1C). Beneficial effect was not observed in the clinical score when VK2 was administered after the onset (from day 11 to 19) for a therapeutic trial (Fig. 1D).

3.2. VK2 reduces the inflammatory cellular infiltration into the spinal cords of EAE

Since there was a prophylactic effect of VK2 on EAE, we next investigated the mechanism of the effect by histological analysis. Spinal cords of vehicle-treated rats showed multifocal intense inflammatory cellular infiltration especially in perivascular areas (Fig. 2A, B). By contrast, spinal cords of VK2-treated rats (in a prophylactic use) exhibited apparently less inflammatory cellular infiltration (Fig. 2C, D). In addition, semi-quantitative evaluation of

![Fig. 2](image_url)

Fig. 2. The inflammatory cellular infiltration into the spinal cords is reduced by the administration of VK2. EAE was actively induced and rats were treated with vehicle alone or VK2 (62.5 mg/kg/day), starting on day –1. The lumbar spinal cords were removed on day 15 and stained with hematoxylin–eosin. (A) Vehicle-treated rats showed multifocal intense inflammatory cellular infiltration especially in perivascular areas indicated by arrowheads. (B) The area surrounded by the square in A was shown in a higher magnification. Severe cellular infiltration with parenchymatous invasion was observed (representative of three animals). (C) VK2-treated rats showed markedly less inflammatory cellular infiltration. (D) Only a small number of perivascular infiltrating cells were observed in a higher magnification (representative of three animals). Scale bars, 200 μm (A, C), and 50 μm (B, D). (E) Mean histological scores of the lumbar spinal cord sections. Three rats from each group were subjected to the analysis, and five sections from each rat were scored according to the criteria described in Materials and methods. Mean histological score in the VK2-treated group was significantly low. Error Bar indicates S.E.M. and data are analyzed with Student’s t-test. *p<0.01.
inflammatory cellular infiltration demonstrated significant reduction of histological score in the VK2-treated group (Fig. 2E).

3.3. VK2 reduces the expression of MHC class II and iNOS in the spinal cords of EAE

Since the expression of MHC class II and inducible nitric oxide synthase (iNOS) is increased and the significance of these molecules in the pathogenesis of EAE has been suggested (Ding et al., 1998; Stuve et al., 2002), we investigated whether the expression of these molecules in the spinal cords of EAE was affected by VK2 treatment. The increased expression of MHC class II was observed in many of the lectin-positive macrophage/microglia and some of the GFAP-positive astrocytes in the spinal cords (data not shown), which was consistent with the previous reports (Gehrmann et al., 1993; Hickey et al., 1985). In contrast to the strong staining with anti-I-A antibody in vehicle-treated group, only a faint staining was observed in the spinal cords of VK2-treated animals (Fig. 3A, B). The expression of MHC class II in the spinal cords was further assessed by Western blot analysis. The intense band of MHC class II was seen in the samples from vehicle-treated rat spinal cords with EAE, while it was not detected in the normal spinal cords of non-immunized rats. The band of the VK2-treated sample was apparently faint compared to that of vehicle-treated group (Fig. 3C). This tendency was confirmed by densitometric analysis (Fig. 3D).

The strong staining with anti-iNOS antibody was observed on the lectin-positive macrophage/microglia especially in perivascular areas, and weak staining was seen on a small number of GFAP-positive cells (data not shown), which is consistent with previous reports (Okuda et al., 1997; Tran et al., 1997). The increased iNOS expression especially seen in round or ameboid shaped macrophage/microglia in vehicle-treated rats with EAE was markedly reduced by VK2 treatment and the immunostaining was scarcely seen (Fig. 4A, B). Western blot analysis revealed that the band of the sample from VK2-treated rats was fainter than that of vehicle-treated group, while the difference was not statistically significant by the densitometric analysis (Fig. 4C, D).

We also assessed the reactive gliosis immunohistochemically. Many of the lectin-positive cells in the spinal cords of VK2-treated rats exhibited ramified form, while many of those in the vehicle-treated rats showed ameboid form (Fig. 5A, B). As for the astrocytes, GFAP-positive cells in the spinal cords of VK2-treated rats appeared less hypertrophic and the processes were shorter than those in the vehicle-treated animals (Fig. 5C, D). Thus, reactive gliosis looked milder in the VK2-treated group, and the expression of both MHC class II and iNOS was reduced.
Fig. 4. The expression of iNOS is inhibited in the spinal cords of VK2-treated rats. (A) Increased expression of iNOS was observed in the lumbar spinal cords of vehicle-treated rats with EAE at day 13. (B) The staining with anti-iNOS antibody was scarcely observed in the spinal cords of VK2-treated rats. Each picture was representative of three animals. Scale bar, 25 μm. (C) Protein extracts from the lumbar spinal cords of rats at day 15 were subjected to Western blot analysis. The band of iNOS of VK2-treated sample was weak compared to that of vehicle-treated rats. The blot is a representative of 4 independent experiments. (D) Quantitative comparison of the expression level of iNOS in the spinal cords. The relative expression of iNOS was reduced in the VK2-treated group, though the difference was not statistically significant. Data are expressed as mean±S.E.M. of 4 independent experiments.

Fig. 5. Reactive gliosis is attenuated by the administration of VK2. (A) Many of the lectin-positive cells exhibited ameboid form in the posterior funiculus of the lumbar spinal cords of vehicle-treated rats with EAE at day 15. (B) In the same region of the spinal cords of VK2-treated rats, the majority of the lectin-positive cells were ramified form. (C) GFAP-positive cells were markedly hypertrophic and had long processes in the lumbar spinal cords of vehicle-treated rats with EAE. (D) In the same region of VK2-treated rats, the staining with anti-GFAP antibody was weaker and the cells were less hypertrophic and had shorter processes (D). Scale bar, 25 μm.
MHC class II and iNOS in those reactive glial cells, especially in microglia, was inhibited by the VK2-treatment.

3.4. VK2 inhibits the expression of iNOS in glial cells in vitro

The experiments in vivo suggested that the expression of iNOS in the spinal cords of rats with EAE was inhibited by the treatment with VK2. This inhibitory effect was investigated in vitro. iNOS was induced in microglial cell line GMI-M6-3 by IFN-γ (10 U/ml) or LPS (0.5 μg/ml), and the IFN-γ induced iNOS expression was enhanced by the costimulation with anti-CD40 antibody (HM40-3, 1 μg/ml). The expression levels of iNOS induced by these stimuli were apparently decreased by the incubation with VK2 and this tendency was confirmed by densitometric analysis (Fig. 6Ai, ii). Though the induction of iNOS in astrocytes by the same stimuli looked weaker than that in microglia, the expression was also inhibited by the incubation with VK2 (Fig. 6B).

Cellular toxicity assay was performed as described in Material and methods to exclude the possibility of toxic effect of VK2 on glial cells, and no significant reduction of the number of live cells was observed (data not shown). Thus, these results suggested that VK2 inhibited the expression of iNOS in both microglia and astrocytes without affecting cellular viability in vitro.

![Fig. 6](image_url)

Fig. 6. VK2 inhibits the expression of iNOS in microglia and astrocytes in vitro. (Ai) Microglial cell line GMI-M6-3 was first incubated with 25 μM of VK2 or the same amount of vehicle for 24 h and then incubated with IFN-γ (10 U/ml), IFN-γ (10 U/ml) plus anti-CD40 antibody (HM40-3, 1 μg/ml), or LPS (0.5 μg/ml) for further 24 h. Then, cell lysates were subjected to Western blot analysis. The expression levels of iNOS induced by these stimuli were apparently decreased by the preincubation with VK2. (Aii) The intensity of the bands was measured densitometrically and iNOS/β-actin ratio is shown. The relative expression of iNOS was significantly reduced by the incubation with VK2. Data are expressed as mean±S.E.M. of 3 independent experiments. *p<0.05. (B) Primary astrocytes were incubated with 25 μM of VK2 or the same amount of vehicle for 24 h and then incubated with various stimuli indicated for further 24 h. Though the induction of iNOS in astrocytes by the same stimuli looked weaker than that in microglia, the expression was also inhibited by the incubation with VK2. Each blot was representative of 3 independent experiments.
4. Discussion

A number of molecules such as inflammatory cytokines, adhesion molecules, and molecules associated with antigen presentation have been suggested to be involved in the development of EAE and MS (Chitnis and Khoury, 2003; Hafler, 2004; Hartung et al., 1995; Huang et al., 2000; Raine, 1994). In this report, we demonstrated the efficacy of VK2 on EAE through the reduced expression of MHC class II and iNOS and the reduced cellular infiltration into the spinal cord.

MHC class II is an essential molecule for antigen-specific immune response and is primarily expressed on antigen presenting cells such as dendritic cells, macrophages, and B cells in immune system, and MHC class II deficient mice are resistant to EAE (Stuve et al., 2002). Increased expression of MHC class II is observed on microglia and astrocytes in the CNS of pathological conditions including MS and EAE, and its significance in their pathogenesis has been suggested (Gehrmann et al., 1993; Hickey et al., 1985; Traugott et al., 1985). HMG-CoA reductase inhibitor atorvastatin inhibits the MHC class II expression on microglia in the CNS of EAE and the beneficial effect on EAE is in part attributed to the down-regulation of this molecule (Youssef et al., 2002). In our experiments, increased expression of MHC class II in the spinal cords of vehicle-treated rats with EAE was markedly inhibited by the treatment with VK2. This inhibitory effect on the MHC class II expression may contribute to the beneficial effect of VK2 on EAE. The expression of MHC class II is inducible by pro-inflammatory cytokines such as interferon-gamma produced by activated T cells. Since the cellular infiltration into the CNS was reduced by the treatment with VK2 (Fig. 2), the reduction of inflammatory cellular migration into the CNS may partly account for the decreased MHC class II expression.

Synthesis of Nitric Oxide (NO) is catalyzed by NO synthase (NOS), which are divided into constitutive form (eNOS and nNOS) and inducible form (iNOS) (Nathan, 1992). The expression of iNOS is increased in microglia and astrocytes in the CNS of EAE as we and others previously reported (Okuda et al., 1997; Tran et al., 1997). The expression correlates with the disease course peaking at acute phase and NO produced via iNOS is suggested to be a pathogenic factor in the induction phase of EAE (Cross et al., 1996; Kopprowski et al., 1993), though it has an inhibitory role in the progression phase of EAE as we previously reported (Okuda et al., 1998). The pathogenic role of iNOS for the development of EAE is supported by the facts that antisense knockdown of iNOS and an inhibitor of iNOS ameliorate EAE (Cross et al., 1994; Ding et al., 1998; Hooper et al., 1997). We demonstrated that VK2 inhibited the expression of iNOS in glial cells in vitro (Fig. 6), and the tendency was also observed in EAE model (Fig. 4). Taken together, these results suggest that the inhibition of iNOS expression by VK2 may contribute to the reduction of clinical severity and inflammation of EAE.

Spinal cords of VK2-treated rats exhibited apparently less inflammatory cellular infiltration as shown in Fig. 2. The inhibition of lymphocyte migration into the CNS by HMG-CoA reductase inhibitor lovastatin has been demonstrated, and the inhibition of Rho isoprenylation in brain endothelial cells is suggested to underlie the mechanism (Greenwood et al., 2003). It is likely that inhibition of Rho activation leads to the inhibition of cell migration and invasion, since Rho plays a crucial role in cell adhesion and migration (Mackay and Hall, 1998). VK2 activates protein kinase A and inhibits the activation of RhoA in hepatocellular carcinoma cell line (Otsuka et al., 2004). Thus, the inhibitory effect of VK2 on RhoA activity might participate in the reduced infiltration of the inflammatory cells in VK2-treated spinal cords in our experiment. However, it remains to be solved that VK2 may have effects on other factors such as adhesion molecules, chemokines, and matrix metalloproteinases, which may affect the cellular migration into CNS.

Reactive gliosis is caused by a number of molecules including inflammation-related molecules such as IL-1, TNF-α, IFN-γ, and NO (Aloisi, 2001; Ridet et al., 1997). Milder reactive gliosis in the spinal cords of VK2-treated rats may be explained by the down-regulation of these molecules by VK2, which may also illustrate the inhibition of the cellular infiltration and of the production of iNOS and MHC class II.

The dose effective for prophylactic treatment of EAE in our experiments (62.5 mg/kg) was nearly 70-fold of that in clinical use. Though we performed a low-dose (2.7 mg/kg) prophylactic trial, beneficial effect was not seen on the clinical score (data not shown). However, VK2 is a safe drug, which has been prescribed for the patients with VK2 deficiency, hypoprothrombinemia, and osteoporosis in Japan almost for 30 years, and the toxic effect of the higher dose of VK2 (250 mg/kg) does not induce untoward effects such as the increase of procoagulant activity and the rate of platelet aggregation in rats (Ronden et al., 1997). Thus, VK2 may be a candidate for clinical application to patients with MS. Since the beneficial effect of VK2 on EAE in our experiments was observed only when it was given prophylactically, VK2 may be applicable for the purpose to suppress the severity of relapse.

In summary, we demonstrate that VK2 attenuates the clinical severity of EAE in a prophylactic manner. Histological analysis, immunohistochemical analysis and Western blot analysis reveal that inflammatory cellular infiltration into the spinal cords is reduced and that the expression of MHC class II and iNOS in glial cells is inhibited by the treatment with VK2. Thus, the beneficial effect of VK2 on EAE may be explained, at least in part, with the inhibition of inflammatory cellular infiltration and the inhibition of the expression of MHC class II and iNOS.
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References


