Etiopathology of Behçet's Disease: Immunological Aspects

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Behçet's disease is recognized as a systemic inflammatory disease of unknown etiology. The disease has a chronic course with periodic exacerbations and progressive deterioration. Previous reports have shown at least three major pathophysiologic changes in Behçet's disease; excessive functions of neutrophils, vasculitis with endothelial injuries, and autoimmune responses. Many reports suggested that immunological abnormalities and neutrophil hyperfunction may be involved in the etiology and the pathophysiology of this disease. HLA-B51 molecules by themselves may be responsible, in part, for neutrophil hyperfunction in Behçet's disease. T cells in this disease proliferated vigorously in response to a specific peptide of human heat shock protein (hsp) 60 in an antigen-specific fashion. T cells reactive with self-peptides produced Th1-like proinflammatory and/or inflammatory cytokines. This leads to tissue injury, possibly via delayed-type hypersensitivity reaction, macrophage activation, and activation and/or recruitment of neutrophils. These data shed new light on the autoimmune nature of Behçet's disease; molecular mimicry mechanisms may induce and/or exacerbate Behçet's disease by bacterial antigens that have activated T cells which are reactive with self-peptide(s) of hsp. This would lead to positive selection of autoreactive T cells in this disease.

Behçet's disease is a systemic inflammatory disease, characterized by recurrent oral aphthosis, genital ulcers, skin lesions and uveitis. Although the etiology of this disease is unknown, various immune abnormalities of Behçet's disease have been observed. In this review, immunological findings in patients with Behçet's disease will be discussed.

Immunological findings of lymphocytes in peripheral blood in patients with Behçet's disease

We and others have found abnormalities in pati-

ents with Behçet's disease similar to those observed in certain autoimmune disorders (Lehner, 1982; Arbesfeld et al. 1988); these include induction of lymphocyte transformation by oral mucosa, cytotoxic effect of lymphocytes on oral mucosa, delayed type hypersensitivity (DTH) skin reaction to skin homogenates, histologic features characterized by an early intense lymphocytic infiltration in oral aphthous ulceration, increase in numbers of CD4+ T cells, CD8+ T cells and γδ T cells (Suzuki et al. 1992; Yamashita et al. 1997), suppressor T cell dysfunction (Sakane et al. 1982), defective IL-2 activity of mitogen-activated T cells (Sakane et al. 1986), increased phenotypically activated or memory circulating T cells (Feron et al. 1992), increased serum concentrations of soluble CD8 and CD25 (Haner et al. 1991) and polyclonal B cell activation (Suzuki et al. 1986).

The coincidence of a unique anatomical localization of γδ T cells with the common mucocutaneous

lesions in Behçet's disease (Hamzaoui et al. 1994), impelled us to investigate the pathogenic role of specific T-cell population(s) in the development of Behçet's disease. We found both phenotypical and functional abnormalities of γδ T cells that are characteristic for Behçet's disease (Yamashita et al. 1997). Despite the general rule that CD45RA and CD45RO are markers for naive and memory cells, respectively, we found that γδ+ CD45RA+ T cells from patients exhibited the characteristic features of memory T cells; and that γδ T cells in patients expressed a unique CD45RA isoform, 205 kd (memory cells) but not 220 kd (naive cells), whereas those from normal donors dominantly expressed CD45RO. The subsequent functional analyses showed that CD45RA+ γδ T cells from patients with Behçet's disease expressed IL-2 receptor β chain and HLA-DR antigen brightly in parallel with the disease activity, and produced much more TNF-α and -β than did CD45RO+ γδ T cells of normal individuals. These γδ T cells in Behçet's disease strongly expressed CD16 and CD56, and produced perforin granules (Fig. 1), indicating phenotypical and functional similarities with NK cells. These data suggested that γδ T cells may play a certain role in

the pathophysiology of Behçet's disease. A remarkable accumulation of γδ T cells is frequently observed in the peripheral blood of Behçet's disease patients, and γδ T cells sometimes constitute more than 60% of the peripheral blood lymphocytes. We also found accumulation of γδ T cells in cerebrospinal fluid from patients having neurogical involvements. Since the central nervous system (CNS) is unrelated to the common distribution of γδ T cells, we should consider a distinct role for γδ T cells in the development of a neurological manifestation in patients with Behçet's disease.

Polyclonal B-cell activation with various autoantibodies to antigens derived from affected organs has been noted in the sera from patients with Behçet's disease (Suzuki et al. 1986). Serum IgA is increased irrespective of the disease activity, while IgG and IgD is elevated during the active phase (Korn et al. 1988). Most of the abnormalities in humoral immunity seem to be related to vasculitis. Elevated circulating immune complex (IC) with the reduction of complement titer is often observed in an early phase of active patients (Gupta et al. 1978). The increased IC may be pathogenic because histologic study demonstrated IC depositions, especially in
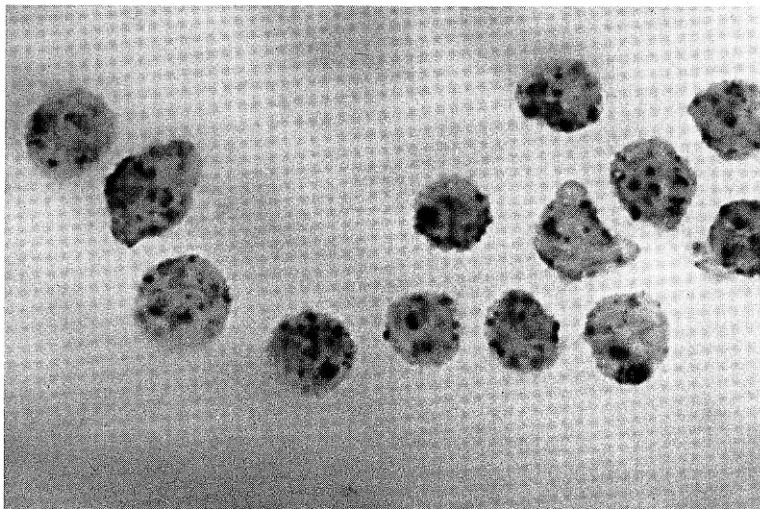
Fig. 1. Immunostaining of perforin granules of γδ T cells in a patient with Behçet's disease. γδ T cells were purified from peripheral blood in patients with Behçet's disease and were stained by anti-perforin mAb, using immunocytochemical method. The amount of perforin granules and the frequencies of perforin-positive cells in γδ T cells in patients with Behçet's disease were greater than those of normal subjects (Yamashita et al. 1997).

HLA-B51 negative patients from whom neutrophils did not reveal excessive chemotactic activity as it did in HLA-B51 positive patients (Sensi *et al.* 1991).

Anti-endothelial cell autoantibodies and anti-cardiolipin antibodies are also thought to contribute to the development of vascular injuries in some patients (de Jesus *et al.* 1996; Toksy *et al.* 1996; Navarro *et al.* 1997). However, unlike vasculitis with other diseases, anti-neutrophil cytoplasmic antibodies are rarely found in patients with Behçet's disease (Burrows *et al.* 1996). Autoantibodies to protein S may be related in the pathophysiology of thrombotic events in patients with Behçet's disease (Guermazi *et al.* 1997). Thus, autoantibodies to components of the coagulation-vascular system may contribute to the development of vascular lesions in patients.

Human heat shock protein (HSP)-60 and Behçet's disease

Recently, analysis of the T-cell receptor (TCR) V α and Vβ gene usage has revealed the oligoclonal expansion of T cells in patients with Behçet's disease; the oligoclonal T-cell expression correlates well with the disease activity (Esin *et al.* 1997). The results suggest a possible role of antigen-specific T cells in the pathophysiology of Behçet's disease.

Reinvestigation of early reports of autoimmune responses to oral epithelial antigen revealed that a 65-kD band was identified with anti-65-kD hsp antibody on Western blots (Lehner *et al.* 1991). Originally, hsp were reported to be involved in the pathophysiology of autoimmune disease. Hsp are unique antigens with a potent immunostimulatory property and they have an extraordinarily high sequence conservation throughout the eukaryotic and prokaryotic kingdoms. 60/65-kD hsp is an immunodominant antigen that is derived from mammalian/bacterial 60/65-kDa hsp and is considered to be a plausible mechanism in the pathophysiology of autoimmune diseases. It has recently been shown that selected peptides derived from the sequences of human 60-kD hsp also induced significant proliferation of T cells in patients with Behçet's disease in England. In addition, two peptides of human 60-kD hsp were most frequently recognized by T lymphocytes from patients with ocular type Behçet's disease. Furthermore, these hsp 60 peptides could induce anterior uveitis in Lewis rats (Stanford *et al.* 1994). We also found that only one hsp peptide (336-351) yielded vigorous proliferation of T cells in Japanese patients with Behçet's disease. We examined TCR usage of T cells responsive to the hsp peptide by means of TCR Vβ subfamily-specific monoclonal antibodies and polymerase chain reaction (PCR) single- strand conformation polymorphism (SSCP)- based technique which enabled us to observe a situation of T-cell clonotypes qualitatively. It has been showed that T cells with specific TCR Vβ subfamilies proliferated in response to the hsp peptide (336-351). Using PCR and subsequent SSCP analysis, the PCR products of freshly-isolated peripheral blood lymphocytes from normal subjects showed a smearing pattern. Interestingly, PCR products of freshly-isolated unstimulated lymphocytes of patients with Behçet's disease resulted in the formation of several distinct bands. Some bands observed in freshly-isolated lymphocytes became prominent after lymphocyte culture with the hsp peptide (336-351) for 9 days in patients with Behçet's disease (Fig. 2). T cells with the same clonotypes proliferated vigorously in response to the same peptide in every uveitis attack in these patients. These data suggest that a molecular mimicry mechanism induces and/or exacerbates Behçet's disease; self-hsp and/or microbial hsp homologous to the self-hsp activates self reactive T cells specific to the hsp peptides. Furthermore, we found that the hsp peptides stimulated oligoclonal T cells producing Th1 cytokines including TNFs, but not Th2 cytokines such as IL-4. Indeed, the plasma level of TNF-α has been shown to be elevated in patients with Behçet's disease as well as increased IFN-γ in the supernatant of T-cell clones derived from cerebrospinal fluid in neuro-Behçet's disease (Parronchi *et al.* 1996). In turn, these Th1 cytokines mediate delayed-type hypersensitivity, prime neutrophils, and stimulate endothelial cells; primed neutrophils and activated endothelial cells interact with each other via adhesion molecules expressed excessively on their surfaces. In addition, neutrophils and endothelial cells may be easily perturbed by subtle stimuli not sufficient to cause lesions in normal subjects, leading to episodic severe symptomatic attacks. Moreover, Lehner recently found anti-hsp65

V β 21 + T cell subfamily

Normal subjects   Patients with Behçet's disease

Fresh   Fresh   the peptide 336-351

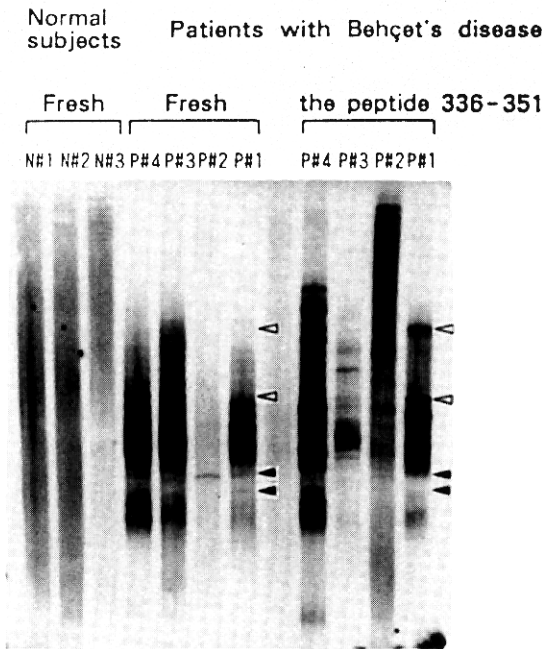N#1 N#2 N#3   P#4 P#3 P#2 P#1   P#4 P#3 P#2 P#1

Fig. 2. TCR clonotype analysis of T cells which react with the hsp peptide 336-351 in patients with Behçet's disease. T cells from normal subjects (N#1, N#2, N#3) and those from patients with Behçet's disease (P#1, P#2, P#3, P#4) were recovered immediately after isolation (fresh) or stimulated for 9 days with the hsp peptide 336-351. (a) The cDNA was amplified by PCR method using TCR Vβ21 specific primers and visualized by SSCP-Southern blotting analysis. After 9 days culture with the peptide, the lower bands (2 open triangles) became prominent whereas upper bands (2 closed triangles) became faint (P#1), suggesting antigen-specific stimulation of the respective T cells (Kaneko et al. 1997).

antibodies in Behçet's disease patients (Direskeneli et al. 1996). Several groups including ours have also demonstrated that lymphocytes from Behçet's disease patients, but neither from normal individuals nor from patients with rheumatoid arthritis, responded to the hsp-derived peptides.

These data shed new light on the autoimmune nature of Behçet's disease; a molecular mimicry between bacterial and self hsp may be associated with the development of the disease; and bacterial hsp may activate self hsp reactive T cells which have been rendered unresponsive by self tolerance

mechanisms. This process would lead to positive selection of autoreactive T cells in Behçet's disease.

Excessive neutrophil function in Behçet's disease

Leukocytosis with neutrophilia and aseptic neutrophilic infiltration into the lesions are characteristic for the active phase of Behçet's disease (Inoue et al. 1994). Furthermore, neutrophilis in patients reveal various abnormalities; upregulated chemotaxis (Matzner et al. 1988), enhanced superoxide synthesis (Niwa et al. 1982), and increased production of chemical inflammatory mediators such as lysosomal enzymes (Hayasaka et al. 1977). Patients' neutrophils express an increased level of adhesion molecules, presumably mediating elevated effector functions and facilitating interaction with endothelial cells (Sahin et al. 1996). All these data suggest a central role of neutrophils in the pathophysiology of the disease.

In accordance with these abnormalities, the therapies for targeting neutrophils such as colchicine are effective in relieving symptoms, further supporting our notion.

The genetic predisposing factor, an HLA-B51 phenotype appears to be associated with neutrophil hyperfunction in Behçet's disease. Chajek-Shaul et al. reported that significantly-enhanced chemotaxis of neutrophils was observed in HLA-B51-positive patients as compared with HLA-B51-negative patients (1987). Sensi et al. demonstrated that superoxide production by neutrophils was elevated in HLA-B51-positive individuals, irrespective of having the disease or not, when compared with HLA-B51-negative individuals (1991). Furthermore, we have shown the excessive neutrophil function in HLA-B51 (B*5101) transgenic (Tg) mice, though the mice lacked Behçet's disease-like symptoms (Takeno et al. 1995). These findings suggest that HLA-B51 molecules by themselves and/or the related gene products induce excessive functions of neutrophils.

Two distinct stimuli are required for neutrophils to generate maximal amounts of superoxides. The first step is priming and the next is triggering. Pure triggering stimuli such as formyl-methionine leucine phenylalanine (fMLP) induce oxidative bursts by primed, but not unprimed, neutrophils. Neutrophils from patients with Behçet's disease, but not normal
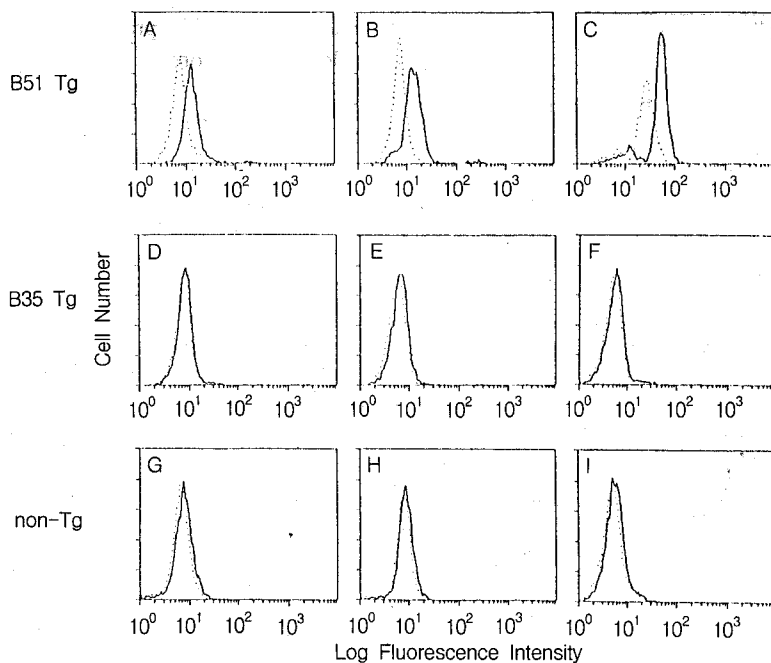
Fig. 3. Superoxide production by peripheral blood neutrophils from HLA-B51 transgenic mice. Superoxide (H_2O_2) production by fMLP-stimulated $(1 \mu g / ml$ for 15 minutes) neutrophils from 3 HLA-B51 transgenic (Tg) (A-C) mice, 3 HLA-B35 Tg (D-F) mice, and 3 non-Tg (G-I) mice. Neutrophil fluorescence was assessed by flow cytometry of stimulated (—) and unstimulated (· · ·) neutrophils (Takeno et al. 1995).

individuals, produced substantial superoxide in response to fMLP, irrespective of their disease activity (Kaneko et al. 1993). Similarly, neutrophils from HLA-B51 Tg mice showed hypersensitivity to fMLP, while HLA-B35 Tg failed to respond (Fig. 3). In contrast, superoxide production induced by phrobol myristate ester and zymosan, both of which do not require the priming process, was comparable between the two Tg mice. It has been shown that once neutrophils are primed, they become refractory to other priming stimuli. Actually, neutrophils from patients with Behçet's disease showed a decreased Ca^{++} influx in response to activated platelet supernatants and ATP, which are both priming stimuli (Sasada et al. 1994). Thus, irritability of the neutrophils in Behçet's disease may reflect that they have already been primed in vivo.

The next question is how the neutrophils in patients with Behçet's disease are primed. Early studies had indicated that patient sera possessed stimulatory effects on neutrophils from normal

individuals, suggesting that some humoral factors such as cytokines and IC are involved in priming neutrophils. Indeed, recent investigation has disclosed increased serum levels of TNF-α, IL -1 β, and IL-8 in patients (Yoshizaki et al. 1995; Eksioglu-Demiralp et al. 1996; Erken et al. 1996; Treudler et al. 1996), irrespective of disease activity. The findings coincide well with the excessive production of cytokines by the peripheral blood lymphocytes observed in vitro (Mege et al. 1993). These findings suggest that excessive proinflammatory cytokines contribute to neutrophil priming, the underlying condition of this disease. Recent studies have confirmed a role for neutrophils as effector cells in an inflammatory response by producing proinflammatory cytokines such as TNF-α, IL-6 and IL-8 (Mege et al. 1993). As expected, neutrophils in patients with Behçet's disease produce elevated levels of TNF-α and IL-8 than do those of normal controls (Nishikawa et al. 1993). Therefore, neutrophils may also contribute themselves via an autocrine loop of

these cytokines.

Vascular injuries with thrombotic tendency are another characteristic feature of Behçet's disease. The damaged or activated endothelial cells are another stimulator to neutrophils. Various abnormalities related to the vascular lesions have been noted in Behçet's disease; the presence of autoantibodies to endothelial cells and cardiolipins (Direskeneli et al. 1995), elevated levels of von Willebrandt-factor antigens and circulating IC (Direskeneli et al. 1995), high plasma endothelin activity (Ural et al. 1994; Uslu et al. 1997), and defective prostacyclin production by endothelial cells (Kansu et al. 1986). In addition, circulating proinflammatory cytokines are involved in activating not only neutrophils but also endothelial cells. Besides the overexpression of adhesion molecules on endothelial cells in the lesions of Behçet's disease, the sera were shown to enhance the production of soluble ICAM-1 from endothelial cells, as well as the expression of Mac-1 and LFA-1 on neutrophils (Aydintug et al. 1995). These phenotypical changes facilitate a series of adhesion molecule-mediated interaction between endothelial cells and neutrophils, leading to acceleration of neutrophil activation. Actually, Inaba et al. demonstrated a prompt shedding of L-selectin on neutrophils in patients with active ocular attack in Behçet's disease (1993), which is recognized as the first step of endothelial

cell-leukocyte interaction during the extravasation of leukocytes in animal models of experimental uveitis. Furthermore, it has been shown that patients sera stimulates endothelial cells to secrete IL-8, which also activates neutrophils in cooperation with adhesion-dependent signaling. Thus, there are multiple factors involved in neutrophil hyperfunction in Behçet's disease: (1) paracrine and/or autocrine proinflammatory cytokines; (2) interaction with activated endothelial cells; and (3) intrinsic abnormalities based on genetic backgrounds. These factors are not mutually exclusive, rather they form a vicious circle to promote the disease.

Characterization of infiltrating cells in skin and ocular lesions in patients with Behçet's disease

Feron et al. reported CD4+ T-lymphocyte involvement in ocular lesions in patients with Behçet's disease by immunohistochemical examination (1992). T lymphocytes which infiltrated ocular lesions were activated in vivo and expressed CD25, one of the activation markers. There were a small number of cells in the optic nerve head, retinal vascular endothelium, and retinal pigment epithelium, all of which were HLA-DR positive (Charteris et al. 1992). They pointed out that interaction between infiltrating CD4+ T lymphocytes and HLA-DR positive cells in
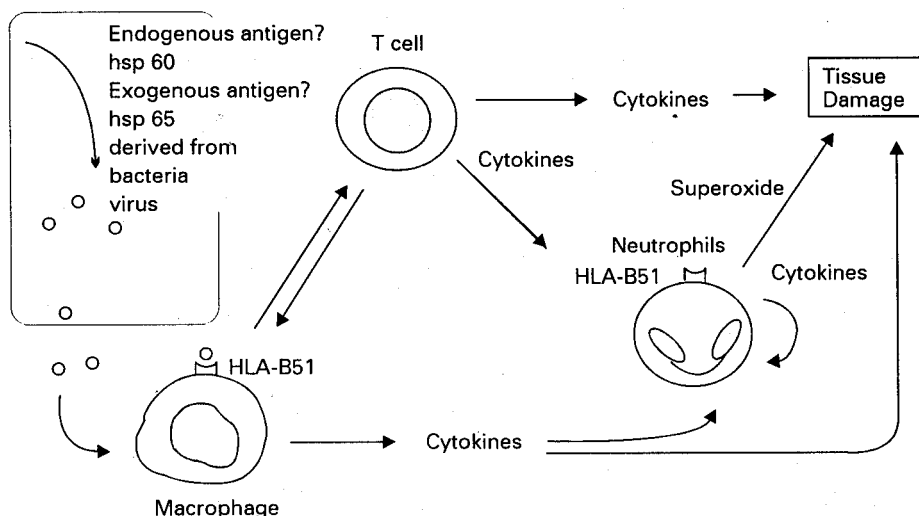

Fig. 4. Schematic representation of the pathogenesis of Behçet's disease.

the eye may induce development of ocular lesions in this disease.

We examined the involvement of T cells and cytokine production in skin lesions in patients with Behçet's disease using immunohistochemical method. We found that CD4+ T cells predominantly infiltrated in skin lesions in patients with Behçet's disease (Yamashita *et al.* 1996). These cells mainly produced IFN-γ, but not IL-4. This data suggested the involvement of Th1 cells in skin lesions in patients with Behçet's disease. We also found that TNF-α, IL-6 and IL-1β-producing cells were accumulating in the lesions. Thus, these proinflammatory cytokines may exacerbate acute inflammation in the skin lesions. Furthermore, we found that human hsp60 expressing cells were prevalent within the lymphocyte-infiltrating area in skin lesions of patients with Behçet's disease. The results suggest that human hsp60 expressed on the skin lesion and hsp-reactive T cells may be intimately associated with the development of Behçet's disease.

The skin pathergy reaction is a non-specific hyperreactive response, which develops at the site of a needle-prick or minor trauma in patients with Behçet's disease, and is very similar to erythematosus papules and pustules appearing spontaneously in these patients. Gul *et al.* examined the immunohistology of skin pathergy reaction in Behçet's disease (1995). A detailed kinetic study of histopathological changes in the site of pathergy tests has revealed that early intense lymphocytic infiltration was followed by persistent neutrophilic accumulation, unlike ordinary inflammatory responses. Rather, T cells appeared to play a dominant role in controlling neutrophil accumulation and their function.

Based on the results discussed above, we hypothesize that there is a relationship between the pathophysiology of Behçet's disease and autoimmune responses present in the disease, as shown in Fig.4. In summary, current reports have suggested the importance of immunologic abnormality in the development of Behçet's disease.

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