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Heat shock protein peptides reactive in patients with Behçet's disease are uveitogenic in Lewis rats

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SUMMARY

Mycobacterial and homologous human heat shock protein T cell peptide epitopes specific for T lymphocytes in Behçet's disease were investigated for their pathogenicity in Lewis rats. The potential pathogenicity of eight peptides and two controls was assessed by administering the peptides in enriched Freund's adjuvant into the footpads of male Lewis rats. Anterior uveitis which is a major manifestation of Behçet's disease was induced with two out of the four mycobacterial and all four homologous human peptides. The most effective peptides inducing iridocyclitis in 64–75% of rats were peptides with amino acids 336–351 and 136–150, derived from the sequence of the human 60-kD heat shock protein. A few of the rats also showed evidence of focal loss of photoreceptors. These results suggest that selected peptides within heat shock protein 60 kD which function as T cell epitopes in Behçet's disease are capable of inducing uveitis in rats. This supports the view that the peptide T cell determinants may be involved in the pathogenesis of Behçet's disease.

Keywords heat shock protein peptides Behçet's disease uveitis Lewis rats

INTRODUCTION

Heat shock proteins (hsp) are highly conserved molecules found in most microorganisms and mammalian cells [1]. HSP have been implicated in the pathogenesis of several human and experimentally induced autoimmune diseases, both as target antigens and as intracellular chaperones involved in peptide binding to HLA antigens [2–5]. Microbial hsp are immunodominant antigens which may be recognized by T cell receptor $\gamma\delta^+$ lymphocytes, and this reactivity appears to be part of an innate rapid response against infection [6,7]. In view of the homology between microbial and human hsp, a breakdown of tolerance to a self-hsp determinant might occur during a microbial infection, resulting in an autoimmune response [8].

Initial evidence supporting a role for microbial hsp in autoimmune diseases was found in the adjuvant arthritis model in rats [9,10]. In humans with reactive and rheumatoid arthritides immunodominant (hsp) T cell epitopes were reported to be involved in these diseases [11–13]. However, further work showed that preimmunization with mycobacterial hsp 60 induced resistance to arthritis in rats, whether induced by Freund's complete adjuvant (FCA) or by unrelated agents [14,15]. These findings implicated hsp 60 both in the induction of arthritis and in some form of suppression that prevented clonal expansion of autoreactive T cells [18]. More recently hsp have been implicated in the pathogenesis of diabetes mellitus, in both the non-obese diabetic (NOD) mouse [17,18] and humans [19]. The mechanism by which immune reactivity against an ubiquitously expressed protein might result in an organ-specific autoimmune disease remains unresolved.

In Behçet's disease, a multisystem disorder of unknown etiology, characterized mainly by recurrent oral and genital ulceration, skin lesions and uveitis, hsp have been implicated in the pathogenesis of the disease [20]. Four separate strains of streptococci have been postulated as etiological agents [21,22], suggesting the possibility of a common shared antigenic determinant [20]. Serum IgA antibodies against mycobacterial 65-kD hsp are significantly increased in Behçet's disease, and a number of MoAbs specific for this protein cross-reacted with selected strains of *Streptococcus sanguis* [20]. Furthermore, polyclonal antibodies against the 65-kD hsp also reacted with a 65-kD band derived from oral mucosa by Western blotting [20]. MoAbs that recognized the 65-kD human mitochondrial hsp also reacted with streptococci and oral mucosa [20]. It is noteworthy that

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increased numbers of circulating T cells bearing the $\gamma\delta$ T cell receptor have been found in Behçet's disease [23,24].

In a recent study we have shown that significant T cell proliferative responses against selected peptides derived from the sequence of both mycobacterial 65-kD hsp and their human synthetic homologues could be generated in lymphocytes from patients with Behçet's disease [25]. This peptide reactivity was not MHC-restricted, and T lymphocytes from the ocular and arthritic types of Behçet's disease were more likely to respond to the peptides than the mucocutaneous type [25]. In particular, lymphocytes from Behçet's patients with eye disease recognized peptides 111–125 and 311–325 of the mycobacterial protein and their human homologous peptides. In order to find out whether peptides recognized by the T cells might have a pathogenic role, we injected these peptides into Lewis rats and examined them for the development of eye disease and for other clinical manifestations of Behçet's disease.

MATERIALS AND METHODS

Peptides and heat shock proteins

Recombinant 65-kD hsp derived from *Mycobacterium bovis* was prepared as described elsewhere [26]. Four synthetic peptides (Table 1) derived from the sequence of the 65-kD hsp that stimulated human T lymphocytes from patients with Behçet's disease, 111–125, 154–172, 219–233, 311–326 and another peptide which did not stimulate these T cells (91–105), were prepared as described elsewhere [25]. Human homologous hsp peptides of the five selected mycobacterial peptides were also prepared (Table 1). The homology between the mycobacterial and the human peptides was as follows: for peptides 136–150, 11/15 residues, for 179–197, 11/19 residues, for 224–258, 9/15 and for 336–351, 10/16 residues were identical or showed conserved amino acid substitutions. The control human peptide 116–130 showed 12/16 residues homology with the mycobacterial peptide 91–105. A computer check through SwissPro and NBRF protein databases showed no sequence homology with retinal S-antigen, rhodopsin, interphotoreceptor retinal binding protein and phosducin, which are known to be uveitogenic [27]. All the peptides were synthesized using endotoxin-free solutions.

Experimental design

A total of 91 age-matched male Lewis rats (Bantin and Kingman, Hull, UK) weighing 200–250 g, were divided into 12 groups. Five hundred micrograms of each of the 10 peptides under investigation were dissolved in PBS and emulsified 1:1 with FCA, enriched with 3 mg/ml killed *Myco. tuberculosis* (strain H37A; Ministry of Agriculture, Weybridge, UK), to a final volume of 200 μl. The emulsion (100 μl) was injected into each hind footpad of rats under ether anaesthesia. Each rat was also given 1×10^{10} heat-killed *Bordetella pertussis* by the i.p. route. This dose of peptide was chosen on the basis of experiments with other synthetic peptides known to induce adjuvant arthritis in Lewis rats [28]. A group of rats was also injected with 500 μg of the recombinant 65-kD hsp, and a control group was injected with the enriched adjuvant mixed with PBS. In order to provide a positive control for uveitis, five rats were injected with 50 μg of purified bovine retinal s-antigen in enriched FCA which was prepared by hydrophobic adsorp-

Table 1. Clinical and histological results of 12 groups of rats injected with five mycobacterial and five homologous human peptides of the 65-kD hsp in enriched Freund's complete adjuvant (FCA)

Peptide		No. of rats	Onset (days)	Duration (days)	Diseases: Clinical *n*	Diseases: Histological* *n*
Mycobacterial peptide sequence						
(111–125)	NPLGLKRGIEKAVEK	10	14–17	2	7	7
(154–172)	QSIGDLIAEAMDKVGNEGV	5	–	–	–	–
(219–233)	LLVSSKVSTVKDLLP	5	–	–	–	–
(311–326)	DLSLLGKARKVVVTKD	10	10–15	3	4	3
(91–105)	ATVLAQALVREGLRN	5	–	–	–	–
65-kD hsp		4	13–18	3	2	2
S antigen		5	10	5	5	5
Enriched FCA		10	–	–	–†	–†
Human peptide sequence						
(136–150)	NPVEIRRGVMLAVDA	11	10–15	3	7	7
(179–197)	KEIGNIISDAMKKVGRKGV	4	11	2	1	3
(244–258)	LLSEKKISSIQSIVP	4	12	2	1	2
(336–351)	QPHDLGKVGEVIVTKD	12	10–14	3	11	9
(116–130)	TATVLARSIAKEGFER	8	–	–	–	–

* All animals showing histological evidence of disease developed predominantly mononuclear cell infiltration of the ciliary body and iris during active disease, and posterior synaechiae after the disease had settled. Two rats treated with peptide (136–150 and 336–351) also showed focal photoreceptor loss and one rat (311–326) developed a vitreous haemorrhage.

† Fibrinous exudate seen clinically only on day 15 was confirmed histologically, but there were no other signs of iridocyclitis.

tion chromatography [29]. All animals were observed clinically from day 7 on a daily basis for signs of uveitis, using slit-lamp biomicroscopy. Animals were killed either during clinical disease or 4 weeks after immunization and their eyes removed for histological examination. The spleen and popliteal lymph nodes were processed for proliferative assays of lymphocytes. The eyes were enucleated and initially fixed in 4% glutaraldehyde for 12 h, slit at the limbus and then immersed in 10% formaldehyde solution. Eyes were dehydrated in graded alcohols, cleared in xylene and paraffin embedded. Sections (5 μm) were cut at 100-μm intervals (10 sections per eye), of which at least one section passed through the optic nerve head, and they were then stained with haematoxylin and eosin.

Lymphoproliferative responses of popliteal lymph nodes and spleen cells

The popliteal lymph nodes were dissected from both hind limbs and the spleen was removed. Each tissue was passed through a stainless steel strainer into RPM1 1640, containing 200 μg/ml penicillin and 200 U/ml streptomycin. The cells were separated on Ficoll–Triosil columns, washed twice in RPMI 1640, and centrifuged at 480 *g* at room temperature. The cells were cultured in 96-well flat-bottomed plates for 5 days in a humidified atmosphere (5% CO_2, 37°C), in RPMI 1640, containing penicillin (100 μg/ml) and streptomycin (100 U/ml) (Gibco, Paisley, UK), and 5% fetal calf serum (FCS; Gibco). Aliquots of 2×10^5 cells/well were cultured with the immunizing peptide at 25, 50 and 100 μg/ml; the hsp65 at 1, 10 and 20 μg/ml, and concanavalin A (Con A) at 20 μg/ml. The cultures were pulsed with 0·5 Ci/well of ^{3}H-TdR (185 Bq/mmol), 16 h before terminating the cultures. They were then harvested using a Dynatech miniwash cell harvester, and ^{3}H-TdR incorporation was determined by liquid scintillation counting. The results were expressed as a stimulation index (SI), being the ratio of ct/min of antigen-stimulated and unstimulated cultures.

RESULTS

Clinical features

Disease usually started in the third week and lasted 2–4 days. Initial signs of eye disease were enlargement and tortuosity of the iris vessels, constriction of the pupil, and the appearance of inflammatory cells in the anterior chamber of the eye (Table 1). In severe cases a fibrinous exudate developed and occasionally a spontaneous hyphaema (haemorrhage in the anterior chamber). The onset of uveitis varied between 10 and 15 days from the time of injection of the peptides, but was slightly later with peptide 111–125 (14–17 days). The duration of the uveitis was between 2 and 3 days. In contrast, S-antigen induced uveitis on day 10 and the lesions lasted 5 days. There was no detectable difference in clinical manifestations of rats injected with the mycobacterial compared with the homologous human peptides or between the different peptides. Late sequelae were not observed in the eye. Clinical involvement was not seen in the mouth, skin or external genitalia.

Histological features

In keeping with the clinical signs, the histological features were moderate in active disease and mild in settled disease. During active clinical disease there was mononuclear cell infiltration in and around the ciliary body and iris (Fig. 1). Polymorphonuclear leucocytes were seen much less commonly. Occasionally, nodular infiltrates of the iris were also observed. When the disease had settled the only consistent histological sign was the presence of posterior synaechiae between the back of the iris and the anterior capsule of the lens (Fig. 2). Pathological signs in the posterior segment were less common; one animal showed a spontaneous vitreous haemorrhage (injected with peptide 311–326), and two animals injected with peptides 136–150 and 336–351 (not in the same experimental group) showed focal photoreceptor loss (Fig. 3). In the control animals only one out of 10 rats showed signs of a mild fibrinous exudate in the anterior segment which was confirmed histologically. All animals injected with S-antigen showed an active endophthalmitis.

Peptides and uvetitis

Clinical and histological evidence of uveitis was not found in any of the rats injected with peptides 154–172, 219–233 (Table 1). In contrast, 7/10 rats injected with peptide 111-125 and 3/10 rats injected with peptide 311–329 showed evidence of

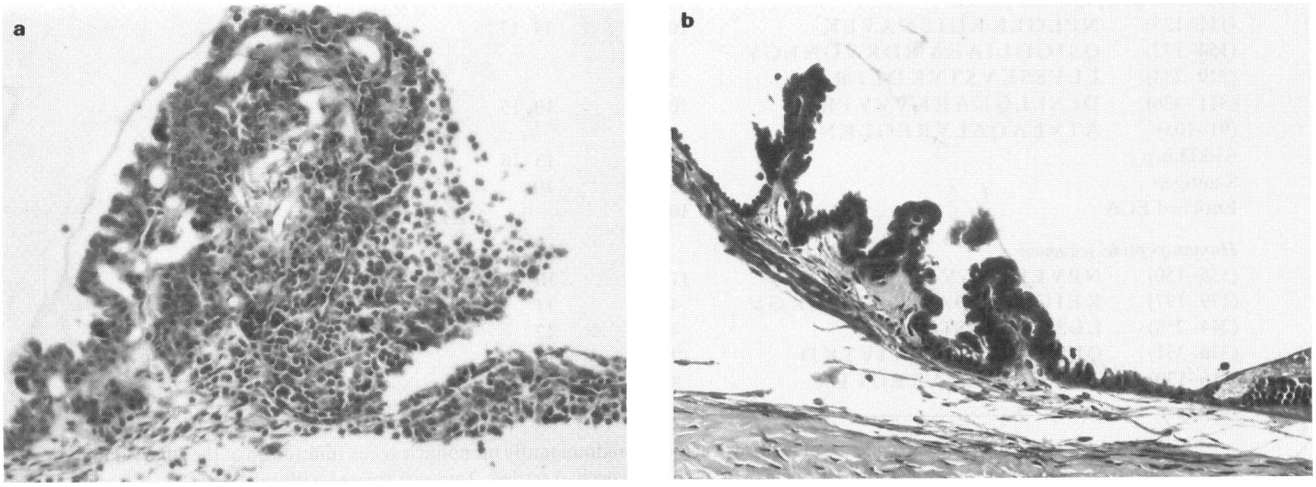

Fig. 1. Photomicrograph of the ciliary body and iris of a rat eye (a) during active disease and (b) in the normal. There is a mononuclear cell infiltration in and around the ciliary body and iris (haematoxylin and eosin × 135).

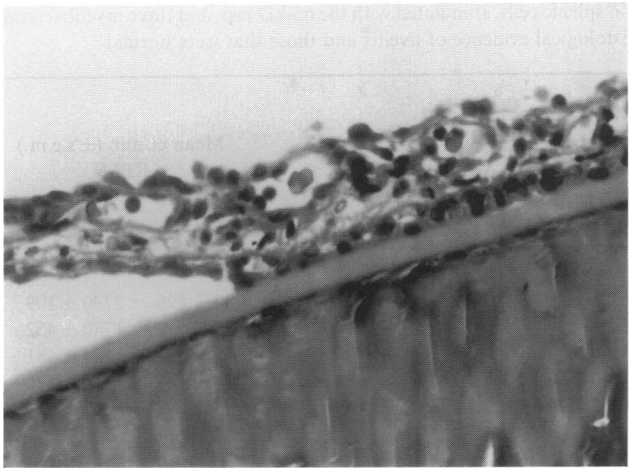

Fig. 2. Photomicrograph of the anterior chamber of the rat eye, after disease had settled, showing posterior synaechiae (attachments between the back of the iris and anterior capsule of the lens), in the absence of any ongoing intraocular inflammation (haematoxylin and eosin × 275).

uveitis. However, all four human homologous peptides induced disease (Table 1); 7/11 (64%) with peptide 136–150 and 9 or 11/12 (> 75%) with peptide 336–351. The other two peptides also induced iridocyclitis, but each peptide was investigated only in four rats, and there was a discrepancy between clinical and histological findings. Neither the control mycobacterial peptide 91–105 nor its human homologue 116–130 induced uveitis in a total of 13 rats. The native recombinant 65-kD hsp caused disease in 2/4 rats. There was no obvious difference in the severity of the disease between the different peptides or the recombinant hsp. The animals injected with bovine retinal S-antigen showed a mixed mononuclear and polymorphonuclear infiltration of the ciliary body, choroid, anterior and posterior chambers, with retinal vasculitis and photoreceptor necrosis.

Lymphoproliferative assays

The popliteal lymph node and splenic cells were cultured with either hsp or the immunizing peptide. The results were analysed separately for rats without uveitis (normal) and those with histological evidence of uveitis (Table 2). There was little difference in SI in rats injected with the mycobacterial peptide 111–125 or its human homologue 136–150, when rats with uveitis were compared with normal rats. However, injection of the human peptide 336–351 elicited higher SI in the popliteal lymph node cells stimulated with the native hsp or peptide 336–351 in rats with uveitis, compared with those without uveitis (Table 2); this difference failed to reach the 5% level of significance when assessed by the χ^2 test. Rats injected with the homologous mycobacterial peptide 311–329 also elicited greater stimulation with the hsp, but not with the peptide in popliteal lymph node cells of those with uveitis, compared with the corresponding normal rats. The control peptides 91–105 and 116–130 induced only a low level of stimulation in a few rats.

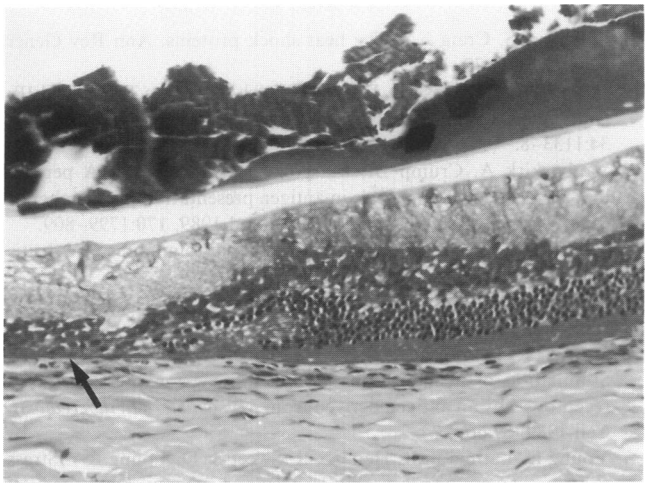

Fig. 3. Photomicrograph of the posterior segment of a rat eye after disease had settled. There is focal loss of the outer retinal layer (arrow), representing photoreceptor cell loss (haematoxylin and eosin × 135).

DISCUSSION

Behçet's disease is a multisystem disorder which is most prevalent in Japan and around the Mediterranean basin, and is associated with high visual morbidity. In an investigation of overlapping synthetic peptides derived from the mycobacterial 65-kD hsp, we reported that $CD4^+$ T cells from patients with Behçet's disease responded to peptides 111–125, 154–172, 219–233 and 311–329 and their human homologous mitochondrial 60-kD hsp peptides [25]. When the patients were divided according to the type of Behçet's disease, those with ocular disease showed the highest frequency of responses, and their T lymphocytes responded especially to peptides 111–125 and 311–326. Furthermore, the homologous human peptides (136–150 and 336–351) were at least as effective as the mycobacterial ones in stimulating T lymphocytes. The results of this investigation suggest that four T cell determinants within the 60–65 kD human or mycobacterial HSP molecule might be implicated in the etiology of Behçet's disease [25].

In the current study we have shown that ocular disease may be induced in Lewis rats by injecting synthetic peptides derived from the sequences of the *Myco. tuberculosis* 65-kD hsp, and with greater frequency by using the homologous human hsp peptides. Mild or moderate clinical disease in these animals appears to be more common in the anterior segment of the eye, and a few animals exhibited spontaneous hyphaemas. Disease onset was usually 10–15 days after injection of the peptides, but occasionally appeared later and usually lasted 2–3 days with no obvious sequelae. Clinical involvement of the mouth, skin, external genitalia, joints or the neurological system was not observed, and the animals appeared healthy. Histological examination of the eyes showed that there was a predominantly mononuclear cell infitration, with a few polymorphonuclear leucocytes affecting the ciliary body and the iris. However, in two rats the posterior segment was involved, with focal loss of photoreceptors. One out of 10 control animals that received adjuvant alone developed mild transient unilateral fibrinous anterior uveitis, the reasons for which were not apparent, since we have not seen this previously, despite extensive experience with this adjuvant. Indeed, 13 other control rats injected with peptide 91–105 or 116–130, mixed with the same adjuvant, were free of any evidence of uveitis. It is unlikely that the uvetitis is part of adjuvant arthritis, as apart from the lack of arthritis, the uveitis in the latter lasts up to 9

Table 2. Lymphoproliferative responses by the popliteal lymph node (PLN) and splenic cells, stimulated with the 65-kD hsp, and three mycobacterial and homologous human hsp peptides in rats which developed histological evidence of uveitis and those that were normal

			Mean SI (± s.e.m.)				Mean ct/min (± s.e.m.) RPMI	
			hsp		Peptide			
Peptide	No.	Histology	PLN	Spleen	PLN	Spleen	PLN	Spleen
(a) *Mycobacterial peptides*								
111–125	4*	Normal	1·78 ± 0·46	1·1 ± 0·10	1·1 ± 0·38	1·3 ± 0·9	1780 ± 696	2790 ± 109
	6	Abnormal	1·9 ± 0·44	2·1 ± 0·42	1·8 ± 0·48	2·2 ± 0·58	833 ± 599	1780 ± 452
311–329	7*	Normal	1·8 ± 0·53	3·16 ± 0·96	0·7 ± 0·14	4·10 ± 0·95	1475 ± 471	1544 ± 481
	3	Abnormal	4·3 ± 1·64	1·1 ± 1·0	1·2 ± 0·31	1·8 ± 1·01	494 ± 114	1316 ± 1260
91–105	5	Normal	2·7 ± 1·32	1·8 ± 0·25	1·1 ± 0·39	2·4 ± 0·61	583 ± 217	3013 ± 374
(b) *Human peptides*								
136–150	5	Normal	1·9 ± 0·46	1·3 ± 0·32	2·6 ± 1·59	1·6 ± 0·32	202 ± 27·0	2055 ± 558·0
	6	Abnormal	1·6 ± 0·52	2·2 ± 0·67	1·1 ± 0·16	1·5 ± 0·15	368 ± 163	2444 ± 236
336–351	2†	Normal	1·4	2·05	1·2	1·0	228	3050·0
	9	Abnormal	3·2 ± 1·5	2·4 ± 0·83	3·4 ± 2·05	0·9 ± 0.10	1146 ± 478	3447 ± 465
116–130	8	Normal	1·0 ± 0·2	1·3 ± 0·08	1·2 ± 0·25	1·3 ± 0·21	514 ± 160	3322 ± 465

* Includes one rat with clinically dilated vessels.
† Both rats had clinically dilated vessels.

days [30] in contrast to the hsp-peptide-induced uveitis, which lasts only 2–3 days.

The peptides that are most frequently recognized by T lymphocytes from patients with the ocular type of Behçet's disease (136–150 and 336–351) produced the highest incidence of disease in the experimental rats. In contrast, both peptide 91–105 and its human homologue 116–130, which are not significant T cell epitopes in Behçet's disease, failed to induce uveitis in rats. Lymphoproliferative responses to the 65-kD hsp or its peptides also showed the highest SI in the popliteal lymph nodes of those rats which developed uveitis when injected with peptide 336–351. However, the SI in rats with uveitis compared with those without uveitis failed to reach the 5% level of significance. The results of these investigations suggest that four synthetic peptides derived from the sequences of human mitochondrial hsp stimulate T lymphocytes from patients with Behçet's disease, and that the same peptides induce uveitis in rats. Although all four homologous mycobacterial peptides can also stimulate T lymphocytes from Behçet's disease, only two of the peptides induced uveitis in rats.

The mechanism leading to uveitis in Behçet's disease is unknown, but the results suggest at least three possibilities: (i) T cell epitopes shared between microbial and human hsp may elicit a cross-reactive immune response which will be directed not only to the microorganism but also against susceptible host cells [8]; (ii) the common T cell peptide determinant may break tolerance to the self-antigen or induce tolerance to the major T cell epitope, allowing a minor T cell epitope to be expressed and give rise to the autoimmune response [31,32]; (iii) hsp or stress proteins are constitutive intracellular proteins which increase with stress, and they may function as chaperones, facilitating folding and assembly of proteins into biologically productive pathways, thereby enhancing the immunogenic or possibly tolerogenic response [33]. However, the mechanism by which single epitopes derived from an ubiquitous mycobacterial hsp give rise to an apparently organ-specific autoimmune disease remains to be determined. These findings might be of some significance, as we know of no other T cell peptide determinant which can specifically stimulate human T lymphocytes from a disease and induce a major manifestation of the disease in experimental animals.

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