

First Cases of Microsporidiosis in Transplant Recipients in Spain and Review of the Literature[▽]

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Microsporidia are currently considered emerging pathogens responsible for life-threatening infections in organ transplant recipients. Here, we describe the first cases of intestinal microsporidiosis by *Enterocytozoon bieneusi* genotype D in two non-HIV-infected renal transplant recipients from Spain. Previously reported cases of microsporidiosis in organ transplant recipients have also been reviewed, highlighting the necessity of considering organ transplant recipients a risk group for microsporidiosis. A systematic search for these parasites is recommended in cases of persistent diarrhea and in the differential diagnosis of other syndromes, such as chronic fever of unknown etiology.

Microsporidia are ubiquitous, obligate intracellular opportunistic parasites, recently related to fungi, capable of infecting a wide range of vertebrate and invertebrate hosts (2, 12). Within microsporidia, 8 genera and 14 species have been associated with human infections, among which *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the most commonly reported (12). These opportunistic pathogens cause a variety of systemic and nonsystemic diseases, with chronic diarrhea as the most common clinical manifestation, although the spectrum of diseases caused by them is broad and includes eye, respiratory, renal, and central nervous system infections (12).

Most microsporidial infections have been reported to occur in severely immunocompromised individuals, mainly HIV/AIDS patients, but cases in HIV-negative people, including travelers and elderly people, are constantly increasing (11, 27). Additionally, the number of non-HIV-infected patients with other forms of immunosuppression is also increasing. Among these, organ transplant recipients (OTR) have recently been considered a group of patients at risk for these pathogens (22). To date, only 21 cases of microsporidiosis in solid organ transplant (SOT) and bone marrow transplant (BMT) recipients who were HIV negative have been described (4, 13–17, 19, 20, 22, 23, 28–31, 34, 40, 44, 49). In these patients, diarrhea is the most frequent clinical manifestation and *E. bieneusi* the species mainly involved (Table 1). Moreover, there are three retrospective studies in which microsporidia have been reported to occur in transplant patients (25, 35, 46). Liguory and collaborators studied microsporidial infection in stool specimens from 100 patients obtained over a 6-year period (1994 to 2000), and

they found 8 organ transplant recipients (6 renal, 1 liver, and 1 heart-lung) who were positive for *E. bieneusi* (25). Rabodonirina and collaborators, in a retrospective study carried out in France, found 23 cases of microsporidiosis in transplant patients, including 3 who had already been described in the literature between 1993 and 2001 (35). Recently, ten Hove and collaborators performed a retrospective phylogenetic analysis on isolates of *E. bieneusi* collected between 2003 and 2004, and they included five kidney transplant recipients that were positive for this microsporidian (46, 47).

We describe the first findings of intestinal infection caused by *E. bieneusi* in two renal transplant recipients from Gran Canaria, Spain, and we review the published cases of microsporidiosis in SOT and BMT recipients.

Case Reports

Patient 1. A 66-year-old male who received a renal transplant in April 2009 was admitted to the nephrology unit of the Hospital Universitario Insular de Gran Canaria (Spain) for severe leucopenia (1,000 cells/mm³) and abundant liquid diarrhea in July of the same year. He had a history of chronic renal failure secondary to a nephroangiosclerosis, arterial hypertension, and a heart attack. Immunosuppressive therapy consisted of steroids, tacrolimus, and mycophenolate mofetil (MMF). This treatment, as well as valganciclovir and septrim, was suspended after admittance to the hospital, due to the suspicion of pharmacologic toxicity. No bacterial or viral pathogens were found in the stool samples. The colonoscopy was normal. Cytomegalovirus (CMV) antigen in colonic mucosa was positive. Calcofluor white (48) and modified trichrome (53) stains showed structures evocative of microsporidial spores in stool samples (Fig. 1). The patient was treated with three doses of filgrastim, fluid therapy, and diet control. The clinical symptoms

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TABLE 1. Microsporidiosis in transplant recipients^a

Case (reference[s])	Type of transplant (no. of patients)	Age (yr)	Gender (no. of patients)	Species/genotype (no. of patients)	Immunosuppressive treatment agent(s)	Clinical manifestation(s)	Laboratory diagnosis method(s)	Treatment/outcome	Country/ Publication yr
1 ^b (25)	Kidney (6); liver (1); heart-lung (1)			<i>E. bienersi</i> /C (7) and D (1)	NA	Diarrhea	MTS, PCR, PCR-RFLP	NA	France/2001
2 ^b (35)	Kidney (14); liver (5); heart and/or lung (4)		F (7); M (16)	NA	ATG, CS, AZ, MMF, tacrolimus	Asymptomatic, diarrhea, wt loss	PCR, TEM	Albendazole, fumagillin	France/2003
3 ^b (46)	Kidney (5)		F (2); M (3)	<i>E. bienersi</i> /C	NA	NA	PCR	NA	Netherlands/2009
4 (40)	Liver	48	F	Undetermined	Tacrolimus, prednisone	Intestinal	MTS	Metronidazole/recovery	U.S./1995
5 (34)	Heart; lung	48	M	<i>E. bienersi</i>	CS, AZ, methylprednisone	Intestinal	MTS, TEM	Metronidazole/recovery	France/1996
6 (20)	Bone marrow	27	F	Undetermined	L-Asparaginase, vincristin, daunomycin, prednisone, CS	Intestinal, respiratory	TEM		India/1997
7 (16)	Kidney	46	M	<i>E. bienersi</i>	AZ, CS, prednisone, MMF	Intestinal	MTS, PCR	Albendazole/recovery	France/1999
8 (16)	Kidney	24	M	<i>E. bienersi</i>	Thymoglobulin, prednisone, CS, AZ, MMF	Intestinal	MTS, PCR		France/1999
9 (17)	Heart	48	M	<i>E. bienersi</i>	CS, AZ, methylprednisone	Intestinal	MTS	Albendazole,	U.S./1999
10 (29)	Kidney	38	F	<i>E. bienersi</i>	Tacrolimus, prednisone, MMF	Intestinal	MTS, PCR	metronidazole/recovery	France/2000
11 (41)	Liver	36	F	<i>E. bienersi</i> /C	Tacrolimus	Intestinal	MTS, PCR	Albendazole/ <i>E. bienersi</i> persistence	Germany/2001
12 (15)	Liver	36	F	<i>E. bienersi</i>	Tacrolimus	Intestinal	MTS, PCR-RFLP	Albendazole/symptomatic improvement, <i>E. bienersi</i> persistence	Germany/2001
13 (23)	Kidney	39	M	<i>Encephalitozoon</i> sp.	AZ, CS, Prednisone	Fever, renal impairment	GCS, TEM	Albendazole/recovery	South Africa/2001
14 (30)	Kidney	43	F	<i>E. cantuati</i> /type III strain	Methylprednisone	Disseminated	GCS, IFAT, TEM, PCR	Albendazole, topical fumagillin (keratitis)/death related to neurological involvement	Canada/2002
15 (31)	Liver	NA		<i>E. bienersi</i>	Tacrolimus	NA	MTS, PCR	Fumagillin/recovery	France/2002
16 (31)	Kidney	NA		<i>E. bienersi</i>	Tacrolimus, prednisone	NA	MTS, PCR	Fumagillin/recovery	France/2002
17 (14)	Kidney	42	M	<i>E. cantuati</i>	Rapamycin, CS, prednisone	Disseminated	IFAT, TEM	Albendazole, fumagillin/relapse 1 year later	Mexico/2003
18 (28)	Kidney	45	F	<i>E. cantuati</i>	Steroids	Disseminated	PCR, TEM	Antimicrobial therapy/death	U.S./2003
19 (33, 45)	Bone marrow	21	F	<i>E. cantuati</i> /type III strain	Thiotepa, CYP, ATG, CS	Pulmonary	MTS, TEM, PCR	NA	U.S./2004-2005
20 (4)	Pancreas; kidney	43	M	<i>Encephalitozoon</i> sp.	Tacrolimus, MMF, prednisone, AZ	Disseminated	TEM	Postmortem diagnosis	U.S./2004
21 (19)	Cornea	60	M	Undetermined	Prednisolone acetate	Keratoconjunctivitis	CW, GS, PCR	Topical ciprofloxacin	India/2006
22 (22)	Kidney	64	M	<i>E. bienersi</i>	Tacrolimus, MMF	Intestinal	Uvitex-2B, PCR		France/2009
23 (22)	Liver	45	M	<i>E. bienersi</i>	CS, everolimus	Intestinal	HE, Uvitex-2B, PCR		France/2009
24 (44)	Kidney	38	F	<i>E. cantuati</i> /type IV strain	Thymoglobulin, MMF, CsA	Disseminated	Uvitex-2B, PCR	Albendazole/recovery	France/2010
Current report	Kidney	66	M	<i>E. bienersi</i> /D	Steroids, tacrolimus, MMF	Intestinal	MTS, PCR	Filgrastim/recovery	Spain
Current report	Kidney	54	M	<i>E. bienersi</i> /D	Tacrolimus, MMF	Intestinal	MTS, PCR	Albendazole, metronidazole/recovery	Spain

^a F, female; M, male; CS, cyclosporine; AZ, azathioprine; MMF, mycophenolate mofetil; CsA, cyclosporine A; Cyp, cyclophosphamide; ATG, antilymphocyte globulin; MTS, modified trichrome stain; RFLP, restriction fragment length polymorphism; GCS, Gram chromotrope stain; HE, hematoxylin-eosin stain; TEM, transmission electron microscopy; IFAT, indirect immunofluorescence assay; PCR, polymerase chain reaction; CW, calcofluor white; GS, Gram stain; NA, not available; ND, not determined.

^b Retrospective study.

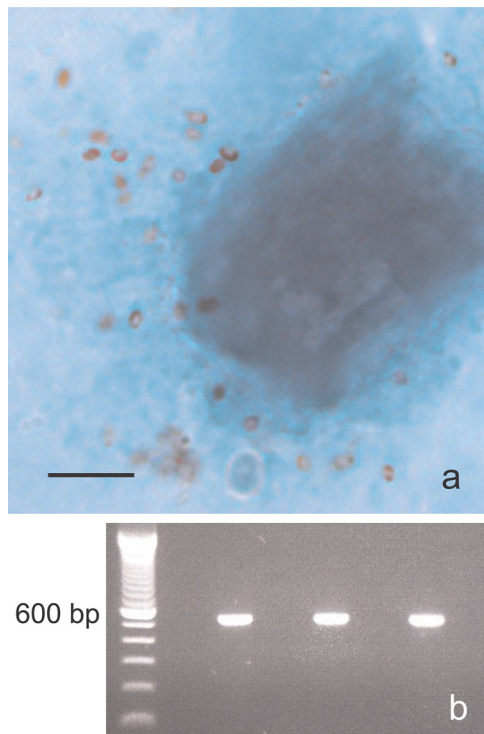


FIG. 1. (a) Microsporidial spores stained with a modified trichrome stain from patient 1. Bar, 5 μm . (b) PCR amplification of the rDNA coding region containing the 243 bp of the ITS of *E. bienersi*. First lane, molecular marker (100-bp ladder); third lane, patient 1; fifth lane, patient 2; seventh lane, positive control; eighth lane, negative control.

disappeared, and the patient was discharged afebrile, with a normal white blood cell count and normal bowel movements.

Patient 2. A 54-year-old man with a history of kidney transplant in 1994 was admitted to the nephrology unit of the Hospital Universitario Insular de Gran Canaria for persistent liquid diarrhea on 9 June 2009. His immunosuppression regimen consisted of tacrolimus and MMF. The patient did not improve after MMF suspension and dietary treatment. Laboratory tests showed a slight deterioration of renal function. Cytomegalovirus antigen was negative. *Clostridium difficile* toxin was detected in fecal samples. Fecal smears showed microsporidial spores stained by calcofluor white (48) and modified trichrome (53) stains. The clinical symptoms disappeared after initiation of fluid therapy, diet control, and metronidazole treatment. Normal bowel habits and renal function were recovered. Two weeks later, the patient showed episodes of diarrhea with epigastric pain, microsporidial spores were observed in stool samples, and results for *C. difficile* toxin, virus, bacteria, and parasites were negative. Treated with albendazole, he became asymptomatic but continued seeding a smaller amount of microsporidial spores, detected only by PCR.

MATERIALS AND METHODS

Staining methods. Thin smears from one diarrheic stool sample from patient 1 and three samples from patient 2 were prepared and stained, using calcofluor white stain (48) and Weber's chromotrope-based stain (53).

DNA extraction and purification. DNA from unpreserved stools was extracted by following the methods described earlier (5). DNA from fecal samples was

extracted by bead disruption of spores using a fast-DNA-spin kit, according to the manufacturer's instructions (Bio 101, Carlsbad, CA). PCR inhibitors were removed using a QIAquick PCR kit (Qiagen, Chatsworth, CA).

PCR amplification. Microsporidial small-subunit-rRNA (SSU-rRNA) coding regions were amplified using the following species-specific primers: EBIEF1/EBIER1 for *E. bienersi* (5), SINTF/SINTR for *E. intestinalis* (6), EHELFL/EHELRL for *Encephalitozoon hellem* (50), and ECUNF/ECUNR for *Encephalitozoon cuniculi* (7). The PCR amplification was done with a GenAmp kit (Perkin-Elmer Cetus, Norwalk, CT) according to the manufacturer's procedures and the conditions for the reaction described previously (8). Purified samples were tested for the presence of PCR inhibitors, as described previously (8). Amplification products were analyzed, electrophoresis was performed with 2% agarose gel, and the samples were visualized by ethidium bromide staining (8).

DNA sequencing analysis. Genotyping of *E. bienersi* was performed by sequence analysis of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). For this purpose, primers that amplified a fragment of 536 bp containing the 243 bp of the ITS were designed (primer F, 5'-CTTCGGCTCTG AATATCTAT3', and primer R, 5'-GCCACTACTAACGGAATCCTA3'). PCR amplifications were performed with the following cycling conditions: denaturing at 94°C for 30 s, alignment at 55°C for 30 s, and extension at 72°C for 90 s. Each PCR product was sequenced in both directions using a BigDye Terminator sequencing kit with an ABI PrismR 3130 genetic analyzer (Applied Biosystems). The resulting sequences were analyzed by the Bioedit program and compared with reference sequences from GenBank.

RESULTS

Staining methods. The two patients studied showed structures evocative of microsporidial spores in the sample analyzed from patient 1 and in two samples from patient 2, obtained before albendazole treatment when analyzed by calcofluor white staining (48) and Weber's chromotrope-based staining (53). Spores in the chromotrope-stained smears appeared pinkish red and measured 0.9 to 1.2 μm in length. Many spores exhibited the characteristic posterior vacuole and beltlike stripe in the middle (Fig. 1a). However, in the second stool sample from patient 2, analyzed 2.5 months later, and after albendazole treatment, no microsporidial spores were observed by the staining methods.

PCR. PCR was performed with unfixed stools from the two patients. Amplification of DNA isolated with specific primers for the most common microsporidia infecting humans showed positive results with *E. bienersi*-specific primers (5) in both cases for all samples. However, *E. intestinalis*-specific PCR (6), *E. hellem*-specific PCR (50), and *E. cuniculi*-specific PCR (7) were negative (Fig. 1b) for the two patients studied.

Genotyping. Genotyping of the *E. bienersi* isolates was performed by sequence analysis of the ITS region of rDNA. The sequence analysis of PCR amplified products showed 100% homology with genotype D in both cases (GenBank accession number AF101200.1) (38).

DISCUSSION

Enterocytozoon bienersi is the most common microsporidian associated with human disease, particularly in severely immunosuppressed individuals with CD4⁺ counts of <100/mm³ (12). In the presence of HIV infection, it is associated with diarrhea and wasting syndrome, and cellular immunoresponse has been considered essential for the control and elimination of this microsporidian (12). In SOT and BMT recipients, an immunosuppressive therapy is always prescribed, leading to a profound cellular immunodeficiency (22). However, few cases of microsporidiosis have been reported to occur in transplant

patients (Table 1). Chronic diarrhea is the main clinical manifestation in most infections and *E. bienewisi* the most common species encountered in more than half of the cases in OTR, followed by *E. cuniculi* (Table 1) (22, 35). This agrees with the observations for the two patients in our study, with persistent diarrhea with *E. bienewisi* detected in stool samples by PCR and a modified trichrome stain. However, microsporidiosis occurred in reported cases from 19 days up to 7 years after transplantation (22). In patient 1 in our study, it appeared 3 months later, but in patient 2, it appeared 15 years after transplantation.

In both patients, we detected other microorganisms that have been associated with gastrointestinal symptoms in transplant recipients, including diarrhea. Patient 1 was positive for CMV antigen in colonic mucosa, which is a common finding, since CMV infection is one of the major infectious complications in transplant recipients with nonsystemic symptoms that include fever, diarrhea, myalgias, malaise, and, in severe cases, hepatitis, pneumonia, and colitis (18). However, the gastrointestinal tract is one of the least common sites of CMV disease. Taking into account that this patient showed no other symptoms besides diarrhea and received prophylaxis with an antiviral, we believe that *E. bienewisi* would play an important role in the diarrhea observed. It should be noted that in one of the renal transplant recipients reviewed, the authors suggest that CMV infection may further enhance the susceptibility to microsporidial infection (30). Patient 2 showed a test positive for *Clostridium difficile*, which is a significant pathogen leading to diarrhea and colitis in transplant recipients (32). However, the majority of *C. difficile* infections concern patients in the early posttransplant period, with a prior long-lasting treatment with antibiotics, which was not the case for patient 2. Nevertheless, the patient was treated with metronidazole, a first-line therapy for *C. difficile* (21), and the results for the *C. difficile* test were negative from then on.

The patients' outcomes were as follows. Patient 1 showed a clinical course similar to that described in previously reported cases for SOT recipients (31), in which suspension of the immunosuppressive treatment led to recovery. Therefore, we suggest that restored immune balance after MMF withdrawal and filgrastim treatment allowed the recovery of the patient. Patient 2 was capable of resolving the symptoms only after a sequential therapy with metronidazole and albendazole: metronidazole treatment initially allowed the elimination of *C. difficile*, and afterwards, albendazole treatment allowed a patient decrease in microsporidial spore seeding and the resolution of diarrheal symptoms. To date, no curative therapy for *E. bienewisi* infection exists. Metronidazole, which is indicated for *C. difficile* treatment (21), has occasionally been reported to cause transient improvement of the symptoms of microsporidiosis (17, 41, 54). However, albendazole, which is effective against microsporidia other than *E. bienewisi*, seems to alleviate diarrhea in *E. bienewisi*-infected patients without clearing the infection, but with a notable decrease in spore seeding (8, 41, 51, 52).

Both patients received immunosuppressive therapy with tacrolimus and MMF at the time of diagnosis. Other immunosuppressive therapies described for OTR diagnosed with microsporidiosis included cyclosporine, prednisone, azathioprine, rapamycin, antilymphocyte globulin, or methotrexate (22, 35).

It has been suggested that the lack of gamma interferon (IFN- γ) resulting from the Th cell depletion induced by MMF may be responsible, at least in part, for the onset of microsporidiosis (16) and the triggering of the intestinal symptomatology by the parasite. Since patient 1 recovered from symptoms after treatment suspension, it is very likely that this recovery was associated with the immune reconstitution balance. The same situation was described in previously published reports of microsporidiosis in renal transplant recipients (16). For patient 2, discontinuation of the immunosuppressive therapy helped by metronidazole and albendazole treatment improved the patient's health conditions, including normal bowel movements. The same outcome has been described to occur in two of the four OTR with *E. bienewisi* infection who were treated with albendazole (34, 41). However, in the other two cases, a complete clearance of spores was achieved (16, 29), suggesting that the discontinuation of the immunosuppressive therapy was probably what mainly improved the patients' health conditions (16, 29, 41).

In reference to the presence of microsporidia in Spain, they have mainly been reported to occur in HIV/AIDS patients (8–10) but also in HIV-negative individuals, including travelers (26), elderly people (27), and the immunocompetent population (1). In most of these studies, *E. bienewisi* was the microsporidian responsible for clinical symptoms. However, to date, no cases of microsporidiosis in OTR have been described. This report recognizes the implication of microsporidia in OTR pathology for the first time in Spain.

In most cases, microsporidia detected in OTR were characterized only to the species level (22, 35). *Encephalitozoon cuniculi* is the best-characterized microsporidian in OTR isolates at the genotype level (30, 33, 44). In three of five *E. cuniculi* infections described to occur in SOT and BMT recipients, the genotype was investigated; two of these infections showed ITS-related genotype III, known as the "dog strain" (30), and for the third infection, a new genotype, genotype IV, was recently described (44). In reference to the genetic variation among isolates of *E. bienewisi* (18, 47), analyses of ribosomal DNA internal transcribed spacer (ITS) sequences have identified more than 70 genotypes of *E. bienewisi* (18, 47). Some of these genotypes have been recognized as host specific, while others have been found to infect humans and animals, supporting the likelihood of zoonotic transmission (18, 47). In our 2 patients, genotype D was identified. This genotype belongs to group 1, which includes numerous genotypes from various origins: humans, both HIV-positive and negative, but also domestic and wild animals. Genotype D is widespread in nature (47). This genotype was first found in humans in Germany and afterwards in other countries of America, Asia, and Africa. It was also found in numerous diverse animals (swine, cattle, macaque, muskrat, raccoon, beaver, fox, dog, and falcon) (47). The type D genotype was commonly reported to occur in HIV-positive patients in Thailand (24) and Peru (42) and in two isolated cases in Europe (36, 37), and it was recently isolated from 3 HIV-negative individuals in Cameroon (3), which confirms the wide spread of this genotype. Genotype D represents 15% of isolates from four species of wildlife animals in North America (43) and 26% of isolates found in cats in Colombia (39), supporting a zoonotic route of transmission for this strain.

Information about molecular epidemiological data for *E. bienewisi* isolates from transplant recipients is limited. Several studies have described the predominance of genotype C in this population (25, 41, 46). Liguory and collaborators (25) genotyped 100 *E. bienewisi* isolates from both HIV- and non-HIV-infected patients, including eight transplant recipients. In the transplant patients, they found genotype II (genotype C) in seven of these patients and genotype IV (genotype D) in one (25). They also described that the distribution of genotypes was significantly different among HIV-infected patients compared to that among non-HIV-infected patients, and they suggested differences in the epidemiology of the infection according to HIV infection status or differences in the virulence of the microsporidial strain (25).

Sing and collaborators also found human genotype C in a liver transplant recipient, based on analysis of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) sequence (41). ten Hove and colleagues performed a molecular characterization of *E. bienewisi* isolates from immunosuppressed and immunocompetent patient groups in Malawi and the Netherlands (46). They identified 16 genotypes, 9 of which had not previously been described. Genotypes B, K, and D were most prevalent among HIV patients, whereas genotype C was identified in five isolates from kidney transplant recipients and was not seen in any of the other groups of patients (46).

In contrast to the microsporidiosis caused by *E. bienewisi*, which is generally confined to the digestive tract, as shown in reported cases (15–17, 29, 31, 34) and in the 2 cases described here, *Encephalitozoon* infections were frequently disseminated in OTR (4, 14, 30, 44, 45). In these cases, microsporidia were most frequently identified in urine samples but were also isolated from various tissues or body fluids, including those from stools, sputum, conjunctival scraping, brain, and kidney biopsy specimens. The most commonly reported clinical symptoms in disseminated microsporidiosis in OTR were keratoconjunctivitis, fever, abdominal pain, and respiratory symptoms (cough and thoracic pain) (44). Diagnosis of microsporidiosis in these patients was conducted mainly by trichrome staining and PCR. Most of the cases were described to occur in Europe, but there were also seven in America, two in India, and one in Africa. The antiparasitic treatment used included albendazole, metronidazole, and fumagillin, independent of the microsporidian species found. In most cases of infection by *E. bienewisi*, the recovery was related to immune reconstitution and/or immunosuppressive therapy suspension (22).

In conclusion, transplant recipients undergoing immunosuppressive therapy should be considered a risk group for acquisition of microsporidiosis. Therefore, in all countries (including those in which microsporidia have not yet been recognized), microsporidia should be considered in cases of persistent diarrhea and also in the differential diagnosis of other syndromes, such as chronic fever of unknown etiology, after more-common causes of diarrheal disease are ruled out. The search should be performed not only in stool samples but also, at least, in urine samples. A molecular characterization of the parasite isolates should be considered to convey information about the frequency and distribution of microsporidian species and genotypes in this group of patients.

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