Recombinant BCG expressing the leishmania surface antigen Gp63 induces protective immunity against *Leishmania major* infection in BALB/c mice

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We have cloned and expressed the *gp63* gene of *Leishmania major* in BCG to develop a recombinant vaccine against zoonotic cutaneous leishmaniasis. Two different expression systems were investigated. The first system consists of *pAN*, a *Mycobacterium paratuberculosis* promoter, which drives expression of ORF2, an open reading frame in IS900. This system allows the production of heterologous polypeptides as hybrids with the ORF2 gene product. The second expression system relies on the production of antigenic fragments as fusion proteins with the N-terminal region of *Mycobacterium fortuitum* β -lactamase. Both constructs resulted in the production of Gp63 in BCG. The ability of the two recombinant BCG strains to induce protective immunity against a challenge with *L. major* amastigotes was evaluated after vaccination of susceptible (BALB/c), and resistant (C57BL/6) mice. Recombinant BCG producing Gp63 as a hybrid protein with the N-terminal region of the β -lactamase elicited significant protection against a challenge with *L. major* in BALB/c-immunized mice.

Keywords: *Leishmania*, Gp63, recombinant BCG, protective immunity

INTRODUCTION

The bacillus of Calmette and Guérin (BCG) is a particularly attractive vector for the development of recombinant vaccines (Bloom, 1989). It has already been used to immunize more than 2·5 billion people with a very low incidence of serious complications and it can be given at or any time after birth. BCG itself is a potent adjuvant: a single inoculum can elicit long-term immunity (Bloom, 1989).

A growing array of integrative and extrachromosomal expression vectors has been developed over recent years, enabling cloning and expression of foreign genes in BCG (Snapper *et al.*, 1988; Ranes *et al.*, 1990; Matsuo *et al.*, 1990). Various expression cassettes have been described for efficient expression of HIV1 Gag and Env structural proteins (Aldovini & Young, 1991; Stover *et al.*, 1991), and the regulatory protein Nef (Winter *et al.*, 1991), tetanus toxin and β -galactosidase (Stover *et al.*, 1991;

Murray *et al.*, 1992) in BCG. Several recombinant BCG strains induce specific cellular and humoral immune responses in mice (Aldovini & Young, 1991; Stover *et al.*, 1991; Winter *et al.*, 1991; Murray *et al.*, 1992). Protective immunity against Lyme disease elicited by recombinant BCG against challenge with *Borrelia burgdorferi* has also been reported (Stover *et al.*, 1993).

Leishmania are obligate intracellular protozoan parasites of macrophages. They cause leishmaniasis, a major infectious disease of considerable public health and economic importance (WHO, 1984). Leishmaniasis includes a broad spectrum of diseases, ranging from simple cutaneous to visceral leishmaniasis (Chang & Bray, 1985), according to the parasite species and to the host's susceptibility.

With the aim of developing a recombinant vaccine against cutaneous leishmaniasis, we cloned and expressed the *gp63* gene of *Leishmania major* (the causative agent) in BCG. Vaccination against cutaneous leishmaniasis is feasible in principle, since individuals who develop the disease acquire long-term immunity against reinfection. Deliberate infection with the virulent cutaneous leish-

Abbreviations: BCG, bacillus of Calmette and Guérin; CL, cutaneous leishmaniasis; i.v., intravenous(ly); s.c., subcutaneous(ly).

manial parasite has been the basis for vaccination programmes in Israel and in countries of the former USSR (Greenblatt, 1980). However, complications associated with such immunization indicated the need for a killed, attenuated or subunit vaccine for CL. Killed *Leishmania* organisms and soluble extracts of promastigotes have been successfully used for immunizing animals (Howard *et al.*, 1984; Liew *et al.*, 1984; Mitchell & Handman, 1983; Scott *et al.*, 1987) and humans (Adler & Gunders, 1964; Lainson & Shaw, 1977; Mayrink *et al.*, 1985). In addition, administration to mice of several purified leishmanial antigens including lipophosphoglycan (Handman & Mitchell, 1985), surface glycoproteins, Gp63 (Russell & Alexander, 1988) and Gp46/M2 (Champsi & McMahon-Pratt, 1988), confers significant protection against leishmaniasis. Effective immunization with these subunit vaccines requires continuous boosters in the presence of an adjuvant. However the same antigen can induce protection against or exacerbation of the disease depending on the route of vaccination (Liew *et al.*, 1985a, b).

Live recombinant vaccines, expressing leishmanial antigens, have been recently investigated as delivery systems for inducing appropriate and long-term immunological responses leading to protection against challenge with *Leishmania* parasites. Different inbred mice strains can be classified as resistant or susceptible to leishmaniasis (reviewed by Blackwell, 1989; Blackwell *et al.*, 1991). Resistant CBA mice orally immunized with the AroA⁻ vaccine strain of *Salmonella typhimurium* transformed with the full-length *L. major* gp63 gene preferentially developed a Th1 response that led to protective immunity against infection with *Leishmania mexicana* (Yang *et al.*, 1990). Susceptible BALB/c mice immunized with recombinant vaccinia virus expressing GP46/M2 were significantly protected against a challenge with *Leishmania amazonensis* (McMahon-Pratt *et al.*, 1993). A recent report (Connell *et al.*, 1993) has shown that BCG expressing Gp63 as a fusion protein with the BCG *hsp60* gene product engendered a protective response against a challenge with *L. mexicana* promastigotes and amastigotes after immunization of CBA and BALB/c mice. This recombinant BCG strain protected resistant CBA mice but not susceptible BALB/c mice against a challenge with *L. major*, although lesion development within this group was less severe than in untreated and BCG control groups (Connell *et al.*, 1993).

We analysed the expression of the *gp63* gene of *L. major* under the control of two different mycobacterial expression systems. The ability of the recombinant BCG-*gp63* vaccines to induce protective immunity against a challenge with *L. major* was evaluated after vaccination of susceptible (BALB/c) or resistant (C57BL/6) mouse strains. One of the expression systems led to significant protection against a challenge with *L. major* in immunized BALB/c mice.

METHODS

Bacterial strains, plasmids, parasites and culture conditions. The bacterial strains, plasmids and parasites used in this study

are listed in Table 1. *Escherichia coli* strains were grown in L-broth supplemented with appropriate antibiotics and were transformed as described by Sambrook *et al.* (1989). Mycobacterial strains were transformed by electroporation as described previously (Ranes *et al.*, 1990). Recombinant BCG clones were first cultivated on Lowenstein-Jensen medium containing 10 µg kanamycin ml⁻¹, then transferred onto Sauton medium for classical vaccine preparation or grown as a dispersed culture (Gheorghiu *et al.*, 1988). *L. major* strain TN435 was cultured in RPMI 1640 supplemented with 10% inactivated foetal calf serum (Hyclone Laboratories) (Jaffe *et al.*, 1984). *L. major* amastigotes were obtained by passaging strain PSA.18.SBZ, isolated from the rodent *Psammomys obesus* from the region of Sidi Bou Zid (Tunisia), and passaged *in vivo* five times in BALB/c mice.

Construction of expression vectors. The Gp63 protein precursor of *L. major* is encoded by an ORF of 1·8 kb, of which 1·5 kb encodes the mature form of Gp63 (Button & McMaster, 1988). The DNA fragment coding for the mature protein was synthesized *in vitro* by PCR amplification from genomic DNA of *L. major* strain TN435 (kindly provided by R. Ben Ismail, Institut Pasteur de Tunis: Ben Ismail *et al.*, 1986). Translational initiation and termination codons, and restriction sites for the subsequent cloning, were added. The sequences of the 5' and 3' primers are 5'-GCGGGATCCTTATGCATGTGCGGACG-TGAAGTGGGGC-3' (the *Bam*HI and *Nsi*II sites are underlined) and 5'-CCGAATTCAAGCTTCTACGCCGTGTTGCGCCGTCCTT-3' (the *Hind*III site is underlined); an ATG start codon and a stop codon were included (in bold).

The *gp63* gene was then cloned in plasmids pAM311 and pIPJ42*. Plasmid pAM311 is a pUC18 derivative containing a 1·6 kb fragment from *Mycobacterium paratuberculosis* harbouring the pAN promoter and ORF2 (Murray *et al.*, 1992). Plasmid pIPJ42* is a pUC18 vector harbouring at the *Bam*HI site the β -lactamase gene *blaF* with the regulatory region of *blaF**, which overproduces β -lactamase (Timm *et al.*, 1994a). pIPJ46 is a pSL1180 derivative harbouring the origin of replication, oriM, of the *M. fortuitum* plasmid pAL5000 (Ranes *et al.*, 1990) and the kanamycin-resistance gene, *aph*, from Tn903 (Kanamycin Resistance Gen-Block, Pharmacia). pIPJ46 was used as a source of the oriMaph cassette for both the *E. coli*/mycobacteria shuttle plasmids, which were named pIPS11 (pAM311 derivative) and pIPS42 (pIPJ42* derivative). These plasmids were introduced into *M. smegmatis* and BCG by electroporation as previously described (Winter *et al.*, 1991).

SDS-PAGE and Western blotting. Total protein extracts of *M. bovis* BCG strains were prepared as described previously (Winter *et al.*, 1991). Proteins were separated on 0·1% SDS-10% (w/v) polyacrylamide gels according to Laemmli (1970). After electroblotting, the membranes were incubated with a monoclonal antibody directed against Gp63 produced in *E. coli* (kindly provided by W. R. McMaster, University of British Columbia, Vancouver, Canada: Button *et al.*, 1991). The membranes were washed and incubated with horseradish-peroxidase-conjugated sheep anti-mouse second antibody and developed with the ECL chemiluminescent substrate according to the manufacturer's instructions (Amersham).

Evaluation of protection against infection with *L. major*.

Four groups of 8–10 BALB/c or C57BL/6 mice were immunized twice at a one-month interval with 10⁶ c.f.u. given intravenously (i.v.) or 10⁷ c.f.u. given subcutaneously (s.c.) of

Table 1. Bacterial strains and plasmids

	Relevant characteristics	Source/reference
Bacteria		
<i>M. smegmatis</i> mc2 155	High-transformation mutant of <i>M. smegmatis</i> ATCC 607	Snapper <i>et al.</i> (1988)
<i>M. bovis</i> BCG	Vaccine strain 1173P2	BCG laboratory collection, Institut Pasteur de Paris
<i>E. coli</i> MC1061	<i>bsdR2 bsdM⁺ bsdS⁺ araD139</i> <i>Δ(ara-leu)₇₆₉₇ Δ(lac)₇₄ galE15 galK16</i> <i>rpsL (Str^r) mcrA mcrB1</i>	Casadaban & Cohen (1980)
Plasmids		
pAM311	pUC18 carrying the pAN promoter and a fragment coding for the N-terminal region of ORF2 from IS900	Murray <i>et al.</i> (1992)
pIPJ46	pSL1180 derivative carrying the oriMaph cassette	Timm <i>et al.</i> (1994b)
pIPJ42*	pUC18 containing <i>blaF</i> under control of <i>pblaF*</i>	Timm <i>et al.</i> (1994b)
Parasites		
<i>L. major</i> TN435	MON25 MHOM/TN/88/TN435	Institut Pasteur de Tunis
<i>L. major</i> PSA.18.SBZ	MON25 MPSA/TN/92/PSA.18.SBZ	Institut Pasteur de Tunis

the BCG vaccine strain 1173P2, recombinant BCG strains IPS42 and IPS11, or no vaccine (naïve mice). One month after the booster injection, mice were challenged in one hind footpad with 2×10^6 amastigotes isolated from the footpad of an infected mouse, as follows. The foot was ground with a cell dissociation sieve-tissue grinder kit (Sigma). Amastigotes were counted and resuspended in PBS at a concentration of 4×10^7 ml⁻¹.

The footpad swelling was estimated by measurement of the foot circumference and subtracting the foot circumference on the day of challenge. At the end of the experiment (120 d after challenge) mice were killed and parasites were recovered from the footpad and counted as described above.

In vitro lymphoproliferative responses to leishmanial antigen. At day 120 after infection with *L. major*, mice were killed, and popliteal lymph nodes draining the cutaneous lesion removed. Mononuclear cells were plated at 4×10^5 cells per well in 96-well tissue culture plates in 200 µl RPMI 1640 (Flow Laboratories) supplemented with 5% (v/v) heat-inactivated foetal calf serum (Hyclone Laboratories), penicillin (100 U ml⁻¹), streptomycin (50 µg ml⁻¹), and 50 µM 2-mercapto-ethanol. Promastigotes from *L. major* strain TN435 were sonicated and centrifuged at 47000 *g* and the supernatant containing soluble leishmanial antigens (Sacks *et al.*, 1987), was used as stimulating antigen at the final concentration of 20 µg ml⁻¹. Cultures were incubated at 37 °C with 5% CO₂, and on day 5, cells were pulsed with 0.5 µCi (18.5 kBq) [³H]thymidine (Amersham) for 18 h. Cells were then harvested and counted with a β scintillation counter. The results are expressed as mean c.p.m. of triplicate cultures and as Δc.p.m. [(mean counts of antigen-stimulated mononuclear cells) – (mean counts of unstimulated mononuclear cells)].

Statistical tests. Data were analysed for statistical significance by using the Mann-Whitney U test, which is the nonparametric version of the two-group unpaired *t*-test. Data were considered significant at *P* < 0.05.

RESULTS

Cloning and expression of *gp63* in BCG

Two different systems were used to drive the expression of *gp63* in BCG. The first system allows the production of

Fig. 1. Western blot analysis of recombinant BCG clones with an anti-Gp63 monoclonal antibody. Lanes: 1, total protein extract from BCG 1173P2; 2, total protein extract from BCG clone harbouring pIPS42; 3, total protein extract from BCG clone harbouring pIPS11; 4, *L. major* promastigote membrane extract.

Fig. 2. Evaluation of protection against *L. major* infection in BALB/c (a, b) and C57BL/6 (c, d) mice immunized subcutaneously (a, c) or intravenously (b, d) with classical (1173P2) or with recombinant BCG. Mean footpad swelling for each mouse group is indicated. The number of mice that lost their footpad or died before the end of the experiment is indicated in parentheses at the end of the respective plot. ●, Naive; ○, 1173P2; □, IPS42; △, IPS11.

heterologous proteins in mycobacteria as fusions with the N-terminus of the protein encoded by ORF2, an open reading frame of 382 codons which belongs to the insertion sequence IS900 isolated from *M. paratuberculosis*. Expression of the fusion is driven by *pAN*, a promoter located upstream of ORF2 (Murray *et al.*, 1992). Previous studies have shown that analogous fusions of the *lacZ* gene to the ORF2 sequence result in the production of fusion proteins with β -galactosidase activity in BCG (Murray *et al.*, 1992). This recombinant BCG elicited T cell proliferative responses and anti- β -galactosidase antibody production in immunized mice.

The second expression system was recently developed for the production and export of heterologous proteins in mycobacteria (Timm *et al.*, 1994a). This system relies on the production of antigenic fragments as fusions with the N-terminal region of β -lactamase (BlaF). Expression is driven by a mutant of the β -lactamase gene promoter (*pblaF**) isolated from an *M. fortuitum* strain over-producing β -lactamase.

The *gp63* gene was synthesized *in vitro* by PCR amplification from genomic DNA of *L. major* strain TN435 and then cloned into plasmids harbouring the expression cassettes described above. The resulting *E. coli*/myco-

bacteria shuttle plasmids were named respectively pIPS11 and pIPS42. These plasmids were introduced into *M. smegmatis* and BCG by electroporation as previously described (Winter *et al.*, 1991).

The production of the fused Gp63 proteins in BCG was assessed by Western blot analysis of total protein extracts using an anti-Gp63 monoclonal antibody (Button *et al.*, 1991) (Fig. 1). The antibody recognized two major products in recombinant BCG strains: 73 kDa and 63 kDa polypeptides, corresponding to the fusion products between Gp63 and, respectively, the N-terminal part of the polypeptide encoded by ORF2 and β -lactamase. The amount of the β -lactamase-Gp63 hybrid was consistently higher than that of the hybrid of Gp63 with the polypeptide encoded by ORF2 (Fig. 1 and data not shown). This is in accordance with recent studies showing that *lacZ* expression in BCG from *pblaF** resulted in a β -galactosidase activity in transformed BCG which was 6 times higher than that from *pAN* (Timm *et al.*, 1994b). The β -lactamase-Gp63 fusion protein was found only in the total extract of the recombinant BCG cells and not in the culture medium (data not shown). This is consistent with previous studies showing that the signal sequence is essential but not sufficient for protein export (Moreno *et al.*, 1980).

Fig. 3. Proliferative responses to soluble leishmanial antigens of popliteal lymph node cells of BALB/c (a) and C57BL/6 (b) mice. Each point represents the proliferative response in Δ C.p.m. for a single mouse receiving either recombinant (IPS42) (A) or non-recombinant BCG (B) or of the control non-immunized group (C), challenged with *L. major* amastigotes. Bars represent arithmetic means of Δ C.p.m.

Protection against *L. major* infection

Four groups of 8–10 BALB/c or C57BL/6 mice were immunized with two injections, at a one-month interval, of BCG or recombinant BCG by two different inoculation routes, i.v. or s.c. A control group of each mouse strain was not immunized. One month after the booster injection the mice were challenged with 2×10^6 *L. major* amastigotes. The development of cutaneous lesions was followed for 11 weeks after infection by measuring footpad swelling (Fig. 2).

The *L. major* strain TN435 caused a progressive disease in BALB/c mice, but a self-resolving disease in C57BL/6 mice (Fig. 2). The ulcerative lesions induced in BALB/c mice by *L. major* strain TN435 were smaller than those caused by other strains described in the literature (8–15 mm). Despite the small size of the lesions, visceralization and severe evolution of the disease was observed, leading to the death of the animals.

BALB/c mice inoculated with BCG producing the β -lactamase-Gp63 fusion protein (IPS42) had significantly ($P < 0.05$) smaller lesions than controls.

IPS42 recombinant BCG administered by either route restricted lesion development. However, i.v. administration was associated with a more rapid regression of the lesion: its diameter regressed significantly ($P < 0.05$) from day 22 (fig. 2). Mouse groups immunized, by the s.c. or the i.v. route, with the non-recombinant BCG (1173P2) vaccine or with the recombinant BCG producing the ORF2-Gp63 fusion (IPS11) developed ulcerous lesions similar in size to those in the non-immunized control group.

C57BL/6 mice which did not receive BCG developed

only a footpad swelling that regressed 6 weeks after challenge with *L. major* amastigotes, as has been reported for other mouse strains resistant to infection with *L. major*. The C57BL/6 mice, immunized s.c. or i.v. with BCG or with either recombinant BCG-*gp63*, developed footpad swelling similar to that in the control group. Lesion development in C57BL/6 mice, naturally resistant to *L. major* infection, was less pronounced only after i.v. immunization with BCG. This is consistent with previous reports describing a protective effect of BCG against *L. major* infection in mice (Fortier *et al.*, 1987; Connell *et al.*, 1993). In contrast to the control group, the footpad swelling of the immunized C57BL/6 mice did not regress completely 8 weeks after challenge. These lesions appeared different from those induced in susceptible BALB/c mice: they were not ulcerated and were free of parasite on day 51 and at the end of the study. A histological examination of the footpad swelling, performed in two mice of these groups, showed a faint interstitial oedema and a subcutaneous infiltrate of lymphocytes with few histiocytic cells. No Leishman bodies were detected.

Evaluation of parasite load and cellular immune responses

The best protection against *L. major* infection was thus obtained in BALB/c mice with recombinant BCG expressing Gp63 as a fusion protein with the N-terminal region of β -lactamase (IPS42). Since the subcutaneous route of immunization appeared promising, we evaluated the cellular immune responses and the number of parasites at the site of challenge in mice injected s.c. during the protocol described above. Three groups of eight BALB/c and C57BL/6 mice received s.c. either IPS42 or non-recombinant BCG or did not receive any vaccine. The mice were then challenged with *L. major* and killed 120 d later. The footpad of each mouse was ground in 2 ml RPMI medium and 20 μ l tissue homogenate was analysed. The presence of one or more parasite per ten $40 \times$ magnification fields was scored as positive. In BALB/c mice immunized subcutaneously with IPS42, parasites were detected in two of eight mice. Five of eight mice immunized with BCG and all eight non-immunized mice were infected. In C57BL/6 mice no parasites were detected in any group studied.

The proliferative responses of lymph node cells were also investigated in the different groups of mice listed above. The cells were stimulated *in vitro* with soluble leishmanial antigen (SLA), as described in Methods. Cells from six of eight mice immunized with IPS42 proliferated vigorously in response to SLA (Fig. 3). In this group the unresponsive mice were those that had persistent cutaneous lesions. Two of six BALB/c mice immunized with BCG developed a lymphoproliferative response to SLA. The responsive mice had no parasites at the end of the study. All six BALB/c mice of the control group had low proliferative responses. In C57BL/6 mice strong proliferation responses specific to SLA were found in all the groups tested.

DISCUSSION

Upon clinical recovery from most forms of leishmaniasis, there is often effective and long-lasting resistance to recurrent disease. Several studies have shown that cell-mediated immunity, rather than humoral antibodies, plays a role in the host defence and resistance to reinfection. For the cutaneous form of leishmaniasis, Th1 subsets of CD4⁺ cells producing γ -IFN are principally host-protective, whereas the Th2 subsets secreting IL-3, IL-4 and IL-5 are disease-promoting (reviewed by Titus *et al.*, 1992).

Several protective antigens have been reported for murine experimental leishmaniasis. One of these antigens, Gp63, is of particular interest since it is expressed in both promastigote and amastigote stages. This antigen is present in all leishmanial species tested (Bordier, 1987) and is highly conserved (Webb *et al.*, 1991). It has been postulated to play a role in the parasite's entry into the macrophage (Russell & Wilhelm, 1986) and its subsequent survival in the phagolysosome through its non-specific proteinase activity (Chaudhuri *et al.*, 1989). Gp63 has been demonstrated to be a protective antigen, but only in the presence of adjuvants or when presented by live recombinant bacteria (Russell & Alexander, 1988; Yang *et al.*, 1990; Connell *et al.*, 1993).

A recent report has shown that BCG expressing Gp63 as protein fusion with the BCG *hsp60* gene product engendered a protective response against a challenge with *L. mexicana* promastigotes and amastigotes after immunization of CBA and BALB/c mice. This recombinant BCG strain protected resistant CBA mice but not susceptible BALB/c mice against a challenge with *L. major*, although lesion development within this group was less than in untreated and BCG control groups (Connell *et al.*, 1993).

In the present study, BALB/c mice were protected against *L. major* infection by a recombinant BCG expressing Gp63 as a fusion protein with β -lactamase. The same antigen expressed as a fusion protein with the IS900 ORF2 gene product failed to induce protective immunity. This might be due to the level of expression of *gp63*, as differences in the expression level of heterologous protein between the two systems have been observed *in vitro* (a factor of 6 in *pblaF**- versus *pAN*-driven expression) (Timm *et al.*, 1994b).

The ability of the *gp63*-recombinant BCG strain to reduce lesion development after *L. major* infection seems to be independent of the route of vaccination: only slight differences in the protective responses were observed between the i.v. or s.c. inoculation routes.

The reduction of lesion size in BALB/c mice immunized with recombinant BCG IPS42 correlated with the absence of parasites at the site of challenge. C57BL/6 mice are resistant to leishmaniasis and develop protective proliferative responses during infection. There is no proliferative response during acute disease in BALB/c mice. We found that a cellular response was induced in BALB/c mice immunized with *gp63*-recombinant BCG. Vacci-

nation with recombinant BCG expressing *gp63* converted the BALB/c mice to a resistant phenotype. Studies are in progress to determine the pattern of cytokine secretion induced by vaccination.

Studies with animal model systems and humans indicate that there is cross-protection between *Leishmania* species that cause the cutaneous forms of the disease (Adler & Gunders, 1964; Lainson & Shaw, 1977). It would therefore be interesting to investigate the effect of recombinant BCG-*gp63* in eliciting a protective immune response against other *Leishmania* species.

Previous studies of protective responses induced by recombinant BCG strains used foreign antigens cloned on pAL5000-derived replicative plasmids. Preliminary studies have shown that recombinant BCG are stable *in vivo*: 1/3 of recombinant BCG harbouring the *lacZ* gene, when injected into mice, showed β -galactosidase activity 3 months after inoculation (Murray *et al.*, 1992). The use of live recombinant BCG as a vaccine will require integrative vectors with strong expression systems allowing the construction of more stable recombinant strains. The present study shows that immunization with recombinant BCG harbouring *gp63* on a replicative plasmid can elicit appropriate immunological responses resulting in protection against infection. This study and others show that recombinant BCG expressing foreign antigens may provide the basis for an effective vaccine against various pathogens.

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