Effects of an anti-exospore monoclonal antibody on microsporidial development *in vitro*

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SUMMARY

In this study we evaluated the effects of the anti-microsporidial exospore monoclonal antibody 3B6, recognizing 3 *Encephalitozoon* species, *Encephalitozoon intestinalis* (Syn. *Septata intestinalis*), *Encephalitozoon cuniculi*, and *Encephalitozoon hellem* on microsporidial growth *in vitro*. Pre-treatment of spores for 24 h with mAb 3B6 resulted in 21–29% fewer infected host cells 4 days after inoculation of the cultures compared to cultures pre-treated with medium or an irrelevant isotype control mAb ($P < 0{\cdot}001$). Fewer intracellular spores ($1{\cdot}2 \pm 0{\cdot}2$) in infected cells were found when mAb 3B6 was present in cultures compared to cultures with medium alone ($4{\cdot}3 \pm 0{\cdot}8$) or an irrelevant isotype control mAb ($4{\cdot}2 \pm 0{\cdot}9$; $P < 0{\cdot}001$). This decrease appeared not to be dependent on time of exposure, mAb concentration, or presence of complement. It is concluded that antibodies, particularly those directed to potential neutralizing-sensitive epitopes on spores, may have a role in the control of microsporidial growth *in vitro*.

Key words: Microsporidia, *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, monoclonal antibodies, *in vitro*.

INTRODUCTION

Microsporidia is a phylum which includes numerous genera affecting many vertebrate and invertebrate species. Recently, several microsporidial genera affecting humans have been found and include *Enterocytozoon*, *Encephalitozoon*, *Nosema*, *Vittaforma*, *Pleistophora*, *Microsporidium* and, more recently, *Nosema*-like and *Trachipleistophora* (Desportes-Livage *et al*. 1985; Didier *et al*. 1991; Cali, Kotler & Orenstein, 1993; Weber & Bryan, 1994; Weber *et al*. 1994; Hartskeerl *et al*. 1995; Cali *et al*. 1996; Field *et al*. 1996; Hollister *et al*. 1996; Sprague & Becnel, 1998). Rarely reported as human pathogens before 1990, the significance of Microsporidia worldwide has become evident (Shadduck & Orenstein, 1993). Several genera have increasingly gained importance as common opportunistic pathogens in immunodeficient individuals, particularly AIDS patients, and also in immunocompetent hosts (Weber & Bryan, 1994; Weber *et al*. 1994; Bryan, Weber & Schwartz, 1997; Enriquez *et al*. 1998). Host immune responses to microsporidia have been studied by some groups (Andreadis, 1985; Cali *et al*. 1996; Didier, 1995; Niederkorn & Shadduck, 1980; Schmidt & Shadduck, 1984) and it may be that antibodies may contribute to controlling microsporidiosis. We developed a monoclonal antibody (3B6) to *Encephalitozoon intestinalis*, *E. cuniculi*, and *E. hellem* spore antigens. By immunoelectron microscopy mAb 3B6 targeted exospore determinants of *Encephalitozoon* spp., and by Western immunoblotting recognized similar protein antigens in *E. intestinalis*, *E. cuniculi* and *E. hellem* (Enriquez *et al*. 1997). We hypothesized that mAb 3B6 may recognize neutralization-sensitive epitopes within microsporidial spores. To test this hypothesis, we conducted experiments to determine whether or not this antibody could alter microsporidial growth *in vitro*.

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MATERIALS AND METHODS

Hybridoma cells

mAb-producing 3B6 hybridomas were maintained in 75 cm^2 flasks (Costar) in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 1% L-glutamine (Gibco Laboratories), 1% sodium bicarbonate and 10% heat inactivated fetal bovine serum (FBS; Gemini Bioproducts, Inc., Calabasas, CA). As previously described (Enriquez *et al*. 1997), hybridoma 3B6 secretes mAb of an IgG2b isotype. An IgG2b isotype control, the mAb-secreting hybridoma designated 92.12, was kindly provided by Dr James Hagler (Western Cotton Research Laboratories). mAb 92.12 recognizes proteins of Boll Weevil (*Coleoptera*: Curculionidae) and exhibited no cross-reactivity with *Encephalitozoon* spp. (Hagler *et al*. 1994).

Animals

Adult 6-week-old male BALB/c mice for ascites production, purchased from Jackson Laboratories

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(Bar Harbor, Maine) and maintained at the University of Arizona Central Animal Care Facility, were allowed to acclimatize for a minimum of 5 days before any experimental procedure. Mice were housed in filter-topped plastic cages with wood chip bedding, and maintained at 12 h photo-period cycles. Food (Agway Prolab Animal Diet Rat, Mouse, Hamster 3000, Agway Inc., Syracuse, New York) and water were provided *ad libitum*.

Ascites production

Ascites for mAb 3B6 and isotype control mAb 92.12 were produced as previously described (Enriquez, Bradley-Dunlop & Joens, 1991), titrated and screened by indirect immunofluorescence assay (IFA) using purified spores. The mAb 3B6 and mAb 92.12 protein concentrations were determined by Ig capture ELISA determined for each sample according to the optical density obtained and from standard graphs of known mAb 92.12 IgG2b concentrations run individually for each ELISA plate.

Parasites

Human isolates of *E. cuniculi* (CDC: V282; De Groote *et al*. 1995) and *E. hellem* (CDC: 0291:V213; Visvesvara *et al*. 1994) were kindly provided by Dr Govinda Visvesvara (CDC, Atlanta, Georgia). An alveolar isolate of *E. intestinalis* (syn. *Septata intestinalis*; Cali *et al*. 1993; Hartskeerl *et al*. 1995) was purchased from ATCC (catalogue no. 50506; Rockville, MD). All isolates were maintained *in vitro* in Vero cells (ATCC) using RPMI-1640 media (Gibco Laboratories, Grand Island, NY) supplemented with 1% L-glutamine (Gibco Laboratories), 1% sodium bicarbonate and 10% FBS. Spores were isolated and purified from cells by size-exclusion chromatography using a Sephacryl S-300 column matrix with a wet bead diameter 40–105 μm (Bio-Rad Laboratories, Hercules, CA and Sigma, St Louis, MO).

Experimental design

Purified *E. cuniculi* spores were incubated with 1 mg/ml mAb at 4 °C for 1, 2, 4, 8, 12 and 24 h. Following washing to remove unbound antibody, 1×10^4 spores derived from each incubation period were plated in wells of 6-well plates containing a sterile round cover-slip in the bottom of each well with 70% confluent Vero cell cultures. *E. cuniculi*/Vero cell cultures were maintained with supplemented RPMI-1640 medium, cover-slips were harvested 4, 12, 24, 48, 96 and 120 h post-culture and stained with Giemsa. There were triplicate cultures for each harvesting time.

To determine the effects of continuous exposures to mAb 3B6 on *E. cuniculi* growth *in vitro*, *E. cuniculi*/Vero cell cultures were maintained with either medium containing 5 μg/ml or 500 μg/ml of mAb 3B6, irrelevant mAb 92.12 at the same concentrations as an isotype-matched control (Hagler *et al*. 1994) or medium alone with or without guinea-pig complement (Sigma). Cultures were maintained by removing 5 ml of old medium and adding the corresponding new medium 3 times a week. Four, 12, 24, 48 and 96 h post-culture cover-slips were harvested and stained with Giemsa. The same design was used with *E. intestinalis* and *E. hellem* cultured in Vero cells. The percentage parasitized cells/field was determined by dividing the number of parasitized cells by the total number of cells/field (of a minimum of 5 fields at 400×) and multiplied by 100.

To evaluate number of spores/cell following antibody treatments, harvested *E. cuniculi* cultures were fixed in 4% glutaraldehyde and stained by IFA using anti-exospore mAb 3B6. Briefly, cover-slips were incubated for 40 min with mAb 3B6 inside a humidified chamber. Following washing, each cover-slip was incubated for 40 min with fluorescein-conjugated goat anti-mouse affinity-purified antisera (Kirkegaard and Perry, Gaithersburg, MD) with Evan's Blue (Sigma). Slides were mounted with DABCO/glycerol solution (Sigma) and spores within and outside Vero cells were counted using an Olympus epifluorescent microscope at 400×. A minimum of 3 fields were evaluated and results were expressed in mean number of spores/cell.

Statistical analysis

Results were analysed by Student's *t*-test. Experimental data were compared to controls which included a culture medium control and the irrelevant IgG2b mAb to Boll Weevil as isotype control (Hagler *et al*. 1994). *P* values of 0·05 or less were considered significant.

RESULTS

Pre-exposure of spores to mAb 3B6 delays growth of E. cuniculi *in vitro*

E. cuniculi spores were cultured with 1 mg of either mAb 3B6 or isotype control mAb 92.12 from 1 to 24 h. As shown in Fig. 1, the percentage of infected cells was reduced when spores were pre-incubated for 24 h with mAb 3B6 as compared to the percentage of infected cells derived from spores pre-incubated with isotype control mAb 92.12 ($P < 0{\cdot}001$). When spores were pre-incubated with mAb 3B6 from 1–12 h prior to culture, there was a similar trend in reduction of the percentage parasitized cells when compared to isotype control mAb pre-treated groups in all days post-culture ($P \leqslant 0{\cdot}01$; data not shown) with exception of 1 and 2 h pre-incubation in cultures harvested 1, 4 and 120 h post-culture ($P \geqslant 0{\cdot}1$).

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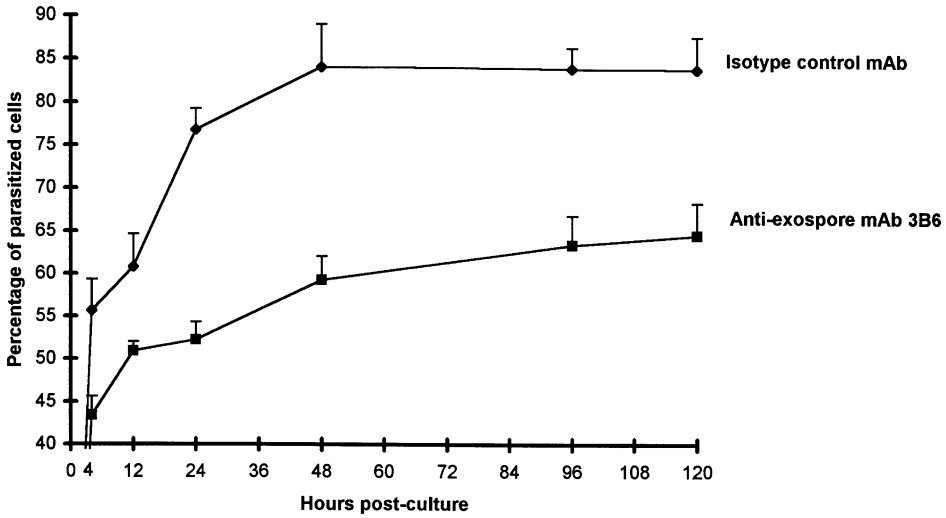

Fig. 1. Percentage of *Encephalitozoon cuniculi*-infected Vero cells following incubation of spores with antibodies. Spores were incubated with mAb 3B6 or isotype control mAb 92.12 for 24 h prior to culture. Cultures, maintained in medium alone, were harvested 4, 12, 48, 96 and 120 h post-culture and the percentage of parasitized cells was determined in triplicate cultures.

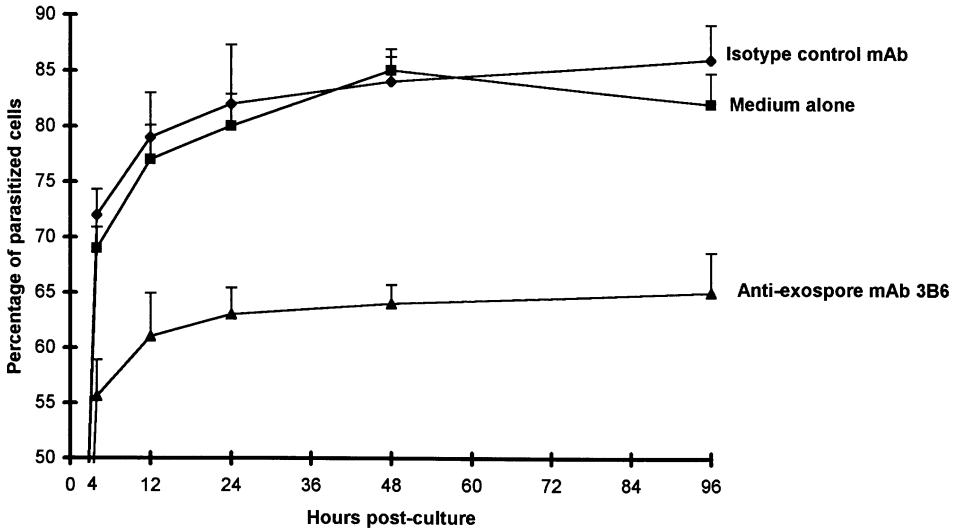

Fig. 2. Percentage of *Encephalitozoon cuniculi*-infected Vero cells in cultures maintained in the presence of antibodies. Cultures were maintained in medium alone (untreated), 5 μg/ml mAb 3B6 or 5 μg/ml isotype control mAb. Cultures were harvested 4, 12, 24, 48 and 96 h later and the percentage of infected cells was determined in triplicate cultures.

mAb 3B6 in culture decreases E. cuniculi *growth* in vitro

To observe whether a continuous exposure to anti-exospore mAb 3B6 would affect microsporidial growth, *E. cuniculi*/Vero cell cultures were maintained with either medium containing 5 μg/ml of mAb 3B6, 5 μg/ml of isotype control mAb 92.12 or medium alone. As seen in Fig. 2, the percentage of parasitized cells decreased significantly when spores were incubated with mAb 3B6 as compared to either the irrelevant mAb isotype control ($P < 0{\cdot}001$) or medium alone ($P \leqslant 0{\cdot}001$) from 4 to 96 h post-culture.

When cells were maintained with 500 μg/ml mAb 3B6, 500 μg/ml isotype control mAb 92.12 or medium alone, there was no significant difference between the percentage parasitized cells in cultures maintained in medium alone and isotype control ($P > 0{\cdot}5$) in all h post-culture studied (data not shown). There was a significant decrease in the percentage of parasitized cells in cultures maintained in 500 μg/ml mAb 3B6 when compared to either medium alone or isotype control mAb ($P < 0{\cdot}001$). This decrease was similar to mAb 3B6 concentration of 5 μg/ml (data not shown).

mAb 3B6 decreases growth of E. intestinalis *and* E. hellem in vitro

E. intestinalis and *E. hellem* growth in Vero cells was significantly decreased when co-cultured with 500 μg/ml mAb 3B6 as compared to isotype control mAb 92.12 or medium alone at 12 ($P < 0{\cdot}05$), 24, 48 ($P < 0{\cdot}01$) and 96 h post-culture ($P < 0{\cdot}001$) with 1 exception: no difference was observed in *E. intes-*

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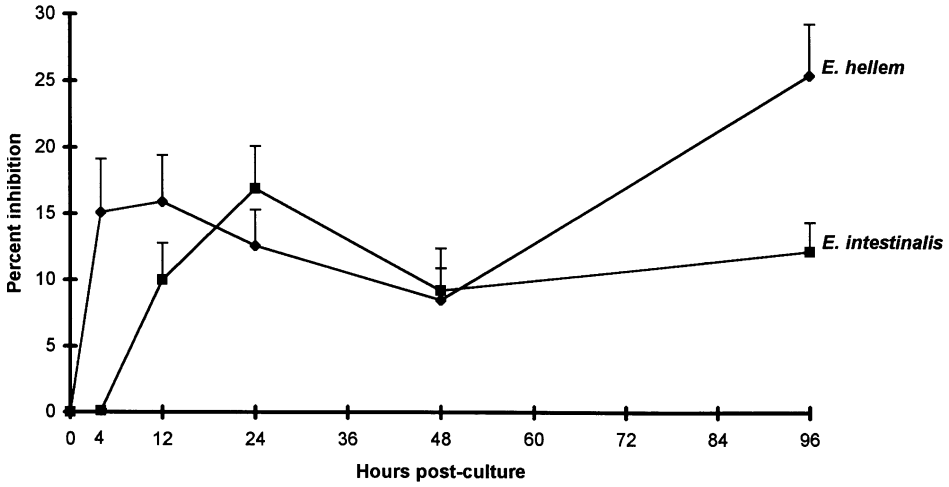

Fig. 3. mAb 3B6-mediated inhibition of *Encephalitozoon intestinalis* and *E. hellem* growth *in vitro*. Cultures were maintained in either 500 μg/ml mAb 3B6 or 500 μg/ml isotype control mAb 92.12. Cultures were harvested 4, 12, 24, 48 and 96 h later and the percentage of infected cells was determined in triplicate cultures. Results are expressed as a percentage inhibition of microsporidia-parasitized cells from isotype control-treated group.

Table 1. Effect of mAb 3B6 and complement *in vitro* on the number of intracellular *Encephalitozoon cuniculi* spores/Vero cell and on *E. cuniculi* growth

Treatment in culture	Mean no. of intracellular spores/cell ± S.D.*	Percentage of parasitized cells ± S.D.†
Medium only	3·8 ± 0·78	83 ± 3·8
Isotype control mAb	3·9 ± 0·91	81 ± 3·8
mAb 3B6	1·6 ± 0·25‡	64 ± 5·9‡
Medium + complement	4·3 ± 0·81	83 ± 1·7
Isotype control mAb + complement	4·2 ± 0·89	82 ± 3·4
mAb 3B6 + complement	1·2 ± 0·16‡	65 ± 5·5‡

* Values are means ± standard deviations (6 samples/group).
† Percentage of parasitized cells was estimated from a minimum of 5 fields at 400 × by dividing the number of parasitized cells by the total number of cells/field and multiplied by 100.
‡ Significantly different from respective values of isotype mAb-treated control group ($P < 0·001$) as calculated by Student's *t*-test.

tinalis growth at 4 h post-culture between mAb 3B6 and control treatments (Fig. 3).

Replication rate of parasites with cells is affected by mAb 3B6

E. cuniculi cultures were treated exactly in the same conditions as in the experiment depicted in Fig. 2. To assess whether the number of intracellular spores differ in the distinct treated and control groups, cultures were fixed and stained by IFA using mAb 3B6 96 h after inoculation. As observed in Table 1 and Fig. 4, the mean number of spores in cells was significantly lower in mAb 3B6-treated cultures as compared to isotype control mAb-treated cultures ($P < 0·001$).

Inhibition of growth is not complement dependent

E. cuniculi cultured in the presence of complement did not differ in the percentage of infected cells when compared to *E. cuniculi* cultures maintained in medium containing heat-treated FBS (Table 1; $P > 0·5$). While co-incubation with mAb 3B6 decreased the growth of *E. cuniculi* when compared to the isotype control mAb ($P < 0·01$), addition of complement did not modify the mAb 3B6-treated percentage of *E. cuniculi*-infected cells when compared to cultures maintained in medium supplemented with heat-treated FBS (Table 1; $P > 0·5$).

DISCUSSION

As in other related protozoan opportunistic infections, functional immune responses to microsporidia may be both cellular and humoral. Immunocompetent and immunodeficient mice, monkeys and humans with microsporidiosis develop parasite-specific antibodies (Niederkorn & Shadduck, 1980;

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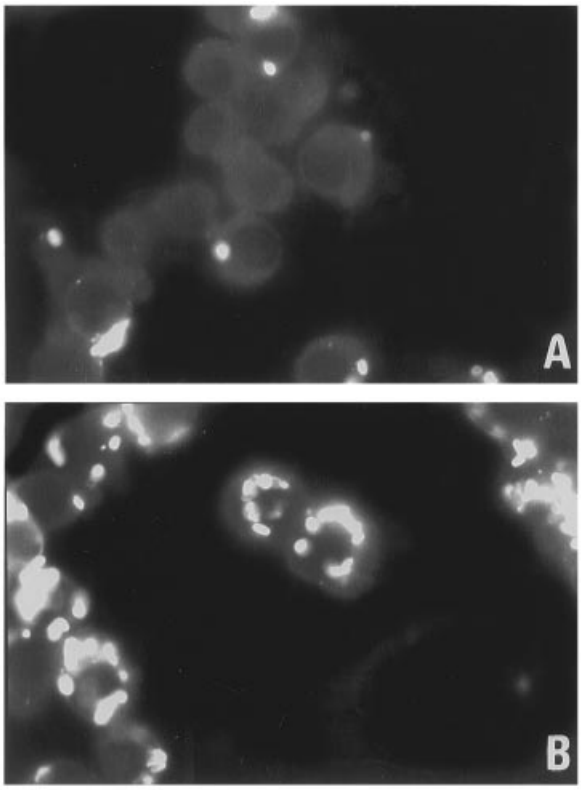

Fig. 4. mAb 3B6-based IFA of *Encephalitozoon cuniculi* cultures maintained in 500 μg/ml mAb 3B6 (A) or 500 μg/ml isotype control mAb (B). Cells were harvested 96 h post-culture, fixed, stained by IFA and examined at 1000× under an epifluorescent microscope.

Gannon, 1980; Bergquist *et al*. 1984*a*, *b*; Schmidt & Shadduck, 1984; Andreadis, 1985; Hollister, Canning & Willcox, 1991; Didier *et al*. 1994; Didier, 1995; Cali *et al*. 1996; Rabodonirina *et al*. 1996; Van Gool *et al*. 1997) and it may be that antigen-specific antibodies within a polyclonal response to infection could contribute to controlling microsporidiosis. In the results herein we observed that pre-incubation or continuous culture of *Encephalitozoon* with mAb 3B6 resulted, to some extent, in growth reduction of *Encephalitozoon* spp. *in vitro*. Since our techniques included light microscopy and IFA, we could not discriminate whether mAb 3B6 affected infectivity on overall microsporidial growth. However, by IFA, the mean number of spores within cells was significantly lower in mAb-treated as compared to isotype control-treated cultures ($P < 0{\cdot}05$). Thus, the replication rate of the parasites might have been affected by mAb 3B6. One explanation for the reduction of microsporidial growth *in vitro* may be that mAb 3B6 targeted neutralization-sensitive epitopes in *Encephalitozoon* sp. spores. It remains to be determined whether spores share epitopes with other intracellular stages. However, it was previously demonstrated that mAb 3B6 recognized developing sporonts (Enriquez *et al*. 1997). The decrease in microsporidial growth in Vero cells *in vitro* appeared not to vary with mAb 3B6 concentrations of 5 and 500 μg/ml. In addition, the mAb 3B6 effect on microsporidial growth *in vitro* appeared not to be complement dependent. While maintenance of cell cultures required addition of fresh medium, the inevitable removal of a few spores may have had an impact on the overall growth curve and could account for some of the variations in growth rate observed at 96 and 120 h post-culture when spores were incubated with mAb 3B6 from 1 to 4 h prior to culture.

The mechanism(s) by which the anti-exospore mAb 3B6 decreases *Encephalitozoon* growth *in vitro* remains to be determined. However, several possibilities may warrant exploration such as effects on limitation or exacerbation of the spores' extrusion of the coiled tubular apparatus or mode of retardation of intracellular growth. One or more of the epitopes recognized by mAb 3B6 may be neutralization-sensitive and it could be that when bound by antibody, spores may be impaired in their ability to inject sporoplasm contents into new host cells. It could also be that sporoplasms were injected into host cells, but because of interaction with mAb 3B6, the intracellular microsporidial growth was retarded in that spores were not killed but opsonized (Didier, 1995).

From the data presented here it is concluded that antibodies may have a role in the control of microsporidiosis, particularly those targeting epitopes on exospore antigens recognized by mAb 3B6 (Mr 34-117). Antibody-dependent neutralization of sensitive epitopes in different microsporidia species may be a host strategy to partially control infections.

We are indebted to Dr Govinda Visvesvara for his kind donation of *Encephalitozoon cuniculi* and *Encephalitozoon hellem*, and to Dr James Hagler for providing the isotype control mAb-secreting hybridoma 92.12. The excellent technical assistance of John D. Palting is greatly appreciated. This work was supported in part by the Fogarty International Center, NIH grant T37-TW00036.

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