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Assessment of epithelial cells' immune and inflammatory response to *Staphylococcus aureus* when exposed to a macrolide

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Non-specific (innate) immune response plays a major role in defending the udder from bacterial invasion. Moreover, recent investigations suggest that mammary gland epithelial cells (MGEC) could have a large and important role as a source of soluble components of immune defences. Despite many attempts to find other ways to control/prevent mastitis (i.e. vaccine) antimicrobial therapy is still the most used and effective means of curing clinical and subclinical mastitis. However, drug concentrations and therapy durations are far from the optimal in order to reduce costs. Therefore, efficacy of antimicrobial therapy is dependent not only on the substance activity but also on the positive interactions with the host innate immune response. Surprisingly, information on these interactions is rather scarce in the mastitis field. A simple experimental model was developed based on BME-UV cell line, *Staphylococcus aureus* as a challenge and a macrolide as an antimicrobial to assess the interactions among epithelial cells, *Staph. aureus* and the potential effects of antimicrobials on the immune system. The results of this study confirmed that tylosin has good antimicrobial activity against both intracellular and extracellular *Staph. aureus* in bovine MGEC without affecting cell functions. In this study, a significant down-regulation of IL-1 and IL-6 was observed, while TNF and IL-8 expression rate numerically increased, but differences were not significant. To our knowledge, this is the first paper assessing the concentration of two lysosomal enzymes, lysozyme and N-acetyl-β-D-glucosaminidase (NAGase), in *Staph. aureus*-stimulated MGEC. The results of this study confirmed that tylosin could have a significant effect on the release of these enzymes. Moreover, even if both enzymes have a similar substrate as a target, the results suggest different secretion mechanisms and an influence of antimicrobial treatment on these mechanisms. Successful mastitis cure is the result of achieving the optimal efficiency of both innate immune defences and therapeutical activities, by means of killing bacteria without eliciting an excessive inflammatory response. Therefore, antimicrobials for mastitis therapy should be selected not only on bacterial sensitivity, but also for their positive interactions with the innate immune response of the mammary gland. This study showed that an in-vitro model based on *Staph. aureus* challenge on MGEC could be helpful in assessing both the intracellular and extracellular activity of antimicrobials and their influence on epithelial cell immune and inflammatory response.

Keywords: Mastitis, macrolides, *Staphylococcus aureus*, immunity, inflammation.

Mastitis is the most economically important disease in dairy herds worldwide, and it is caused by more than 100 bacterial species, even if several risk factors, including host susceptibility are relevant in the epidemiology of the disease.

Non-specific (innate) immune response plays a major role in defending the udder from bacterial invasion (Zecconi & Smith, 2003; Rainard & Riollet, 2006). Udder immune response has similar characteristics to the lung immune response. Indeed, innate cellular [polymorphonuclear neutrophils (PMN) and macrophages] and soluble immune defences [cytokines, lysozyme, lactoferrin, defensins and N-acetyl-β-D-glucosaminidase (NAGase)] are all involved in preventing bacterial adhesion, multiplication and in bacteria killing (Zecconi & Smith, 2003; Burton & Erskine, 2003). Moreover, recent investigations suggest that mammary gland epithelial cells (MGEC) could have a

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large and important role as a source of soluble components of immune defences (Piccinini et al. 2007; Rainard & Riollet, 2006).

Despite many attempts to find other ways to control/prevent mastitis (i.e. vaccine) antimicrobial therapy is still the most used and effective means of curing clinical and subclinical mastitis. However, drug concentrations are largely lower and treatments have a shorter duration in food-producing animals in comparison with the ones applied in human medicine. Therefore, efficacy of therapy is dependent not only on the antimicrobial activity but also on the positive interactions with host innate immune response. Surprisingly, information on these interactions is rather scarce (Nickerson et al. 1985; Zecconi et al. 1996; Hoeben et al. 1997; Zecconi & Piccinini, 2001).

The availability of stabilized cell lines such as BME-UV developed by Zavizion et al. (1996) allows the exploring of the interactions between bacteria and MGEC, including the innate immune response and opens the way to assess also the role of antimicrobials on both bacteria and MGEC (Didier & Kessel, 2004; Fitzgerald et al. 2007). Thus, a simple in-vitro model applying BME-UV cells as a substrate, *Staphylococcus aureus* isolates as challenge and tylosin as treatment was developed. *Staph. aureus* is the major contagious pathogen worldwide (Zecconi & Piccinini, 2002; Zecconi, 2007) and it can adhere and invade MGEC (Lammers et al. 1999; Dego et al. 2002). Macrolides are antimicrobials characterized by having a 12- to 16-member lactone ring and they are classified according to the number of atoms of the ring. Tylosin is a natural macrolide of the 16-ring class, isolated from *Streptomyces fradiae*, and it is a weak base and highly soluble in lipids. Macrolides such as tylosin inhibit protein synthesis by binding to 50S ribosomal subunits and are bacteriostatic (Giguere, 2007). Macrolides penetrate the cellular membrane and accumulate within the cells, as shown also for tylosin (Scorneaux & Shryock, 1999). There is increasing evidence of their anti-inflammatory and immunomodulating properties, at least for the molecules used in human medicine (Zalewska-Kaszubska & Gorska, 2001; Labro, 2004a,b; López-Boado & Rubin, 2008). Among macrolides, tylosin is one of the most used molecules for mastitis therapy both in lactation and in the drying-off period (Ziv, 1980b; Bolourchi et al. 1995; Zecconi et al. 1999; Bonnier et al. 2006). Some information on the interactions between tylosin and leucocytes is available, but not for other macrolides used in mastitis therapy (Zecconi et al. 1996; Chin et al. 2000; Zecconi & Piccinini, 2001; Cao et al. 2006). Based on this information we selected tylosin to assess its potential activities on *Staph. aureus*-infected MGEC.

The aim of this paper was to assess the potential effects of antimicrobials on the immune system when invading bacteria are present, by applying a simple experimental model based on the BME-UV cell line, *Staph. aureus* as a challenge and a macrolide as antimicrobial. The BME-UV cell line was selected because it expresses immunological and inflammatory molecules; *Staph. aureus* because it is an intra- and extra-cellular mastitis pathogen and tylosin because it has an intra- and extra-cellular activity against Gram-positive mastitis pathogens.

Materials and Methods

Bacteria characteristics

Ten *Staph. aureus* isolates from subclinical mastitis cases in dairy cows of different herds were considered. Isolates were selected from our strain collection based on their genetic characteristics and antimicrobial susceptibility. Indeed, they have different virulence gene patterns (*efb, spa, cna*) and leukocidin genes (*pvl, hla, lukDE, lukM*) as described elsewhere (Zecconi et al. 2005; Zecconi et al. 2006). The minimum inhibitory concentration (MIC) for tylosin was assessed for each isolate by means of the agar dilution method following the guidelines of the Clinical and Laboratory Standards Institute (NCCLS, 2000; NCCLS, 2002). Tylosin for these assays and for the experiment was provided by Elanco, Eli Lilly (Italy).

Epithelial cells

A clonal cell line (BME-UV) established from udder primary epithelial cell synthesizing several milk components (Zavizion et al. 1996) was used as an in-vitro model. Previous reports show that this cell line can express several cytokines when stimulated by bacteria or toxins (Didier & Kessel, 2004; Fitzgerald et al. 2007).

Experimental design

BME-UV cells were exposed to different *Staph. aureus* isolates in the logarithmic growth phase at a final concentration of 10^5 CFU, assessed spectrophotometrically, and incubated in a CO_2 incubator at 37 °C for 2 h to allow cell invasion by bacteria. Each isolate was assessed separately. Then, tylosin at a concentration of 1/3 of the MIC (1/3×), the MIC (1×) and 3-times the MIC (3×) of the respective isolate, was added to the infected cell line and incubated at 37 °C for 14 h in a CO_2 incubator. The antimicrobial was used at different concentrations to assess the presence of a dose-dependent effect and the influence of viable bacteria count. At the end of the incubation time, supernatant and cells were collected to assess bacteria count and for biochemical and molecular assays.

Sample preparation

At the end of the incubation period cell monolayer supernatants were pipetted into sterile plastic tubes and 1000 µl used for bacteria counts, while 1000 µl was stored at −20 °C for biochemical assays. To lyse extracellular bacteria, 40 µl of lysostaphin (Sigma-Aldrich, Italy) in 500 µl of HBSS (Sigma-Aldrich, Milan, Italy) was added to each

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Table 1. Description of primer used in amplification of bovine GAPDH gene and cytokine genes

Primer	Length	Forward	Reverse	Source
GAPDH	119 bp	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAGT	Leutenegger et al. 2000
IL-1	116 bp	CTGTTATTTGAGGCTGATGACC	TTGTTGTAGAACTGGTGAGAAATC	†
IL-8	105 bp	CACTGTGAAAATTCAGAAATCATTGTTA	GAAGGTTGTGCAGGTATTTGTGAAG	Leutenegger et al. 2000
TNF-α	103 bp	TCTTCTCAAGCCTCAAGTAACAAGT	CCATGAGGGCATTGGCATAC	Leutenegger et al. 2000
IL-6	163 bp	CACTCCAGAGAAAACCGAAGC	GAAGCATCCCGTCCTTTTCCTC	†

† Designed from NM_174088 *Bos taurus* sequence

sample and incubated at 37 °C for 30 min to destroy *Staph. aureus* adhering to cells. Then, the cell monolayer was trypsinized and the cell suspension was centrifuged at 700 g for 10 min. Pellet samples used for cytokine assays were stored at −80 °C, while pellet samples used for enzyme assays and bacteria counts were suspended in 500 µl of saline solution and stored at −20 °C.

Bacteria count

Extracellular and intracellular *Staph. aureus* counts were performed by the standard dilution method. One-hundred µl of sample dilutions (up to 10^{-3}) were plated on blood agar plates and incubated at 37 °C for 18 h.

Cytokine assays

Total RNA was extracted from cells with RNAqueos kit (Ambion Inc., Austin TX, USA), while reverse transcription was performed with Quanti Tect kit (Qiagen, D). Real-time PCR systems for bovine GAPDH and the cytokines were run in triplicate with probes described in Table 1 and commercially available master mix for Sybr green (Power Sybr Green Master Mix, Applied Biosystems, Foster City CA, USA). Real-time Q-RT-PCRs were performed by using the Opticon 2 detection system (BioRad, Milan, Italy) and expression rate was calculated with Rest 2005 software (Pfaffl et al. 2002). Cytokine expression was assessed as expression rate compared with a reference represented by BME-UV cells not exposed to *Staph. aureus* and not treated with tylosin. A preliminary trial showed that without bacterial challenge and in the presence of tylosin, changes in cytokine expression rates were not detectable. Therefore, a unchallenged tylosin-treated sample was not included in the experiment.

Biochemical assays

Lysozyme (LYZ) was assessed in duplicate by a fluorescence-based procedure (EnzChek Lysozyme Kit, Invitrogen, Carlsbad CA, USA). The method is based on lysis of *Micrococcus lysodeycticus* labelled with fluorescine to such a degree that fluorescence is quenched. LYZ activity is measured by changes of fluorescence on a microplate fluorimeter at 355 nm exc and 460 nm em (Ascent, Thermo Labsystem, FL) against a standard curve obtained for each test with a range of 8–500 units. One unit of LYZ is defined as the quantity of enzyme that produces a decrease in turbidity of 0·0001 OD units per min at 450 nm measured at pH 7·0 (25·8 °C) using 0·3 mg/ml. NAGase (NAG) was assessed in duplicate by the procedure described by Kitchen et al. (1978) and expressed as units (pmol of 4-methylubelliferon released per min at 25·8 °C catalysed by 1 ml of milk) on a microplate fluorimeter at 355 nm exc and 460 nm em (Ascent, Thermo Labsystem, FL).

Statistical analysis

Staph. aureus virulence patterns were clustered by the UPGAMA method and clusters were defined by the presence of a relatedness <80% between isolates as described by Piccinini et al. (2008).

Statistical analysis on response variables was performed by GLM procedure of SAS software (SAS rel 9.2, Cary NC, USA) with bacteria counts, cytokine expression rates and enzyme concentrations as response variables and treatment, bacteria and virulence factors clusters as independent variables.

Results

MIC values of *Staph. aureus* isolates were in the range 0·625–5·0 mg/l. All the isolates were positive for *hla* gene, while for the other genes different patterns were observed. However, statistical analysis did not show any significant influence of isolates on the response variables. Therefore, they were not considered further.

Antibacterial activity

All *Staph. aureus* isolates were found intracellularly, independently of the exposure to antimicrobial treatment. Analysis of antimicrobial activity measured in the supernatant of the cell cultures (Table 2) showed a numerical linear decrease of bacteria concentration as tylosin concentration increased, with significant differences among 1/3× and the other two treatment levels (1× and 3×).

The same pattern was observed for intracellular killing of *Staph. aureus* (Table 2) with an increasing killing rate as tylosin concentration increased. Differences among treatment groups were always significant ($P<0·05$).

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Table 2. Extracellular and intracellular *Staphylococcus aureus* mean counts (±SD) of BME-UV cells exposed to different levels of tylosin (see text for details of concentrations) for 14 h

Staph. aureus	Tylosin 1/3×, $\log_{10}$ CFU/ml	Tylosin 1×, $\log_{10}$ CFU/ml	Tylosin 3×, $\log_{10}$ CFU/ml
Extracellular	$4{\cdot}52 \pm 1{\cdot}06^{a}$†	$3{\cdot}16 \pm 0{\cdot}38^{b}$	$2{\cdot}55 \pm 0{\cdot}58^{c}$
Intracellular	$4{\cdot}51 \pm 0{\cdot}51^{a}$	$3{\cdot}92 \pm 0{\cdot}44^{b}$	$3{\cdot}49 \pm 0{\cdot}49^{b}$

†Mean values in a row with different letters are statistically different ($P<0{\cdot}05$)

Cytokine expression

Analysis of IL-1 expression rate (Table 3) showed a significant reduction of IL-1 in tylosin-treated samples when compared with the untreated sample, without any significant difference among treatment groups.

Analogously, the expression rates observed for IL-6 (Table 3) were significantly ($P<0{\cdot}05$) reduced in tylosin-treated cells when compared with matching untreated controls, with significant differences among treatment groups.

When TNF expression rate was considered (Table 3) the pattern observed was reversed in comparison with IL-1 and IL-6 with rather small increases of expression rates in treated samples, in comparison with untreated controls. However, none of the differences observed was statistically significant. The same pattern was observed for IL-8 (Table 3) but with a large increase of expression rate in treated samples, without significant differences.

Lysosomal enzymes

When lysosomal enzymes were considered, a significant increase ($P<0{\cdot}05$) in intracellular NAG (Table 4) was observed in tylosin-treated cells, when compared with untreated control. However, no differences were observed among treatment groups. Extracellular NAG values were much lower than intracellular ones and without significant differences among groups.

Lysozyme concentration was significantly lower both intracellularly and extracellularly, when compared with untreated controls (Table 4). In both cases the differences between control and respective treatment groups were statistically significant, while significant differences among treatment groups were not observed.

Discussion

In the past, leucocytes were considered the main source of molecules involved in inflammatory and immunological responses. It has been shown that other cells could be an important source of inflammatory and immunological mediators. Indeed, lung epithelial cells could modulate the inflammatory response in the airways and modulate cell recruitment through producing chemokines, cytokines,

Table 3. Relative mean expression rate (±SD) of cytokines in BME-UV cells exposed to *Staphylococcus aureus* and to different levels of tylosin (see text for details) compared with untreated and unexposed controls

Cytokine	*Staph. aureus* unexposed, folds	Tylosin 1/3×, folds	Tylosin 1×, folds	Tylosin 3×, folds
IL1	$11{\cdot}9 \pm 9{\cdot}4^{a}$†	$0{\cdot}7 \pm 0{\cdot}5^{b}$	$1{\cdot}5 \pm 2{\cdot}1^{b}$	$1{\cdot}2 \pm 1{\cdot}3^{b}$
IL6	$14{\cdot}3 \pm 10{\cdot}1^{a}$	$1{\cdot}0 \pm 0{\cdot}6^{b}$	$1{\cdot}2 \pm 0{\cdot}8^{b}$	$1{\cdot}8 \pm 1{\cdot}9^{b}$
IL8	$7{\cdot}4 \pm 4{\cdot}0^{a}$	$16{\cdot}5 \pm 21{\cdot}8^{a}$	$20{\cdot}2 \pm 23{\cdot}9^{a}$	$17{\cdot}1 \pm 19{\cdot}8^{a}$
TNF	$0{\cdot}9 \pm 0{\cdot}5^{a}$	$1{\cdot}8 \pm 1{\cdot}8^{a}$	$2{\cdot}1 \pm 2{\cdot}4^{a}$	$2{\cdot}7 \pm 2{\cdot}9^{a}$

†Mean values in a row with different letters are statistically different ($P<0{\cdot}05$)

receptors and adhesion molecules (López-Boado & Rubin, 2008). Similarly, udder epithelial cells could produce both cytokines and immunomodulating molecules (Didier & Kessel, 2004; Rainard & Riollet, 2006; Fitzgerald et al. 2007; Piccinini et al. 2007). Thus, the role of MGEC cannot be ignored in performing mastitis pathogenesis and therapy studies, and particularly when the interactions between bacteria and host are of interest.

Antimicrobials are still the most used tool to control and cure clinical and subclinical mastitis worldwide, and their efficacy is usually associated with their direct effect on bacteria. However, the efficacy of antimicrobial therapy for mastitis should not only be related to direct antimicrobial activities but also to its interactions with the host immune system. This is particularly true when bovine mastitis treatment is considered, because the quantity and the length of the treatments are respectively lower and shorter than optimal levels, to limit the cost of the treatment and milk withdrawal time (Ziv, 1980a; Wagner & Erskine, 2006).

Although there are plenty of papers on antimicrobial activity in mastitis treatment very few addressed the interactions with the immune system (Nickerson et al. 1985; Zecconi et al. 1996; Hoeben et al. 1997; Zecconi & Piccinini, 2001). These kinds of studies are indeed difficult and expensive; thus, the availability of in-vitro models to assess both the interactions between bacteria and epithelial cells and the immune/inflammatory response could be helpful to assess in a holistic way antimicrobial activity. We applied an in-vitro model, designed to mimic as closely as possible what happens in a mammary gland when a new infection occurs and milk leucocytes are few and not activated. Therefore, MGEC were used as a substrate, different *Staph. aureus* isolates able to invade cells represented the challenge and the incubation time of 14 h resembles the interval between milkings, when both bacteria grow and antimicrobial works. In this model a macrolide, tylosin, was used as a prototype to assess interactions of antimicrobials with MGEC because macrolides are known to be active both intra- and extra-cellularly and to have immune/anti-inflammatory effects in man.

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Table 4. Mean intracellular and extracellular concentration (±SD) of NAGase and lysozyme in BME-UV cells exposed to *Staphylococcus aureus* and to different levels of tylosin (see text for details)

Enzyme	Location	*Staph. aureus* no tylosin	Tylosin 1/3×	Tylosin 1×	Tylosin 3×
NAGase, Units	Intracellular	74·3±20·6[a]†	324·0±117·2[b]	286·1±103·8[b]	327·1±99·7[b]
	Extracellular	51·9±9·0[a]	66·9±4·7[a]	63·1±5·4[a]	67·3±6·5[a]
Lysozyme, Units	Intracellular	32·6±12·6[a]	6·5±12·9[b]	5·3±11·3[b]	8·7±16·2[b]
	Extracellular	86·4±25·8[a]	10·0±28·3[b]	0·0±0·0[b]	0·0±0·0[b]

† Mean values in a row with different letters are statistically different ($P<0·05$)

All the *Staph. aureus* isolates were shown to be able to invade epithelial cells in a very short time and to multiply, even when a sub-optimal dose of antimicrobial was applied. An influence of isolate characteristics on cellular response to the invasion and on antimicrobial treatment was expected, but the statistical analysis did not show any significant results. These unexpected results could be explained by the relatively high dose of inoculum used (10^5 CFU) or by a weak relationship between virulence pattern considered to classify isolates and their competence to invade cells.

The present results confirmed that tylosin had no effects on cell viability and functions. Moreover, a statistically significant antimicrobial activity against both intracellular and extracellular *Staph. aureus* in BME-UV cells was observed, confirming previous data on milk leucocytes (Zecconi et al. 1996). Therefore, our data suggest that when intracellular bacteria are involved, antimicrobials with intracellular activity could be helpful in improving bacteria clearance.

Previous studies show that macrolides, including tylosin can have an anti-inflammatory/immunomodulating effect by inhibiting the prostanoid pathway and by down-regulating pro-inflammatory cytokines production, such as TNF, IL-1, IL-6, 6-keto-$PGF1_a$, and NO (Ianaro et al. 2000; Cao et al. 2006). In our study, we observed a significant down-regulation of IL-1 and IL-6, while TNF and Il-8 expression rates numerically increased, but differences were not significant. Previous studies were performed using mainly leucocytes, and observing these effects also in epithelial cells supports the presence of the anti-inflammatory/immunomodulating activity of tylosin. It is worth noticing that the absence of a down-regulation of chemotactic chemokine IL-8, which would affect the cellular response, can be considered a positive outcome.

To our knowledge, this is the first paper assessing the concentration of two lysosomal enzymes, lysozyme and NAGase, in *Staph. aureus*-stimulated MGEC. Even though both enzymes have a similar substrate as a specific target for their activity, the results obtained suggest different secretion mechanisms. This was not completely unexpected because differences were also observed in NAG and LYZ concentrations in bovine milk of healthy animals (Piccinini et al. 2007). In the present study, extracellular NAG levels were not affected by treatment, but a significant increase was observed intracellularly. Both intracellular and extracellular LYZ levels were significantly decreased in tylosin-treated cells, without differences among treatment group. Both enzymes have acetyl-glucosamine as a target. This molecule is a component of bacterial cell walls, but it is also heavily involved in several biochemical pathways in Golgi apparatus and lysosomes (Cooper & Hausman, 2007). It is well known that LYZ activity plays a role in the host immune defences by killing ingested bacteria in the phagolysosomes and by the control of colonization through exocytosis (Zecconi & Smith, 2003). In this latter case, the killing activity is related to the damage of bacteria cell walls rich in acetyl-glucosamine, and to other enzymic means not yet completely investigated (Ganz, 2004). NAGase is a glycosidase known to be produced in tubular epithelial cells. In this latter case, tubular dysfunction led to an increased release of this enzyme, and therefore, it is considered as a marker of inflammation in kidney diseases (Bazzi et al. 2002). It is also detectable in milk and its levels are correlated with stage of lactation in healthy cows (Piccinini et al. 2007) or inflammation (Kitchen et al. 1984). The patterns observed in the present study support the suggestion that this enzyme is much more involved in intracellular biochemical pathways (Cooper & Hausman, 2007) than in a direct antibacterial activity, even if its release could be enhanced by cellular dysfunctional status (Bazzi et al. 2002) or bacterial stimuli (Kelly & Carchman, 1987).

The different concentration pattern of the two enzymes confirms that tylosin treatment reduces the inflammatory response, as suggested by the significant decrease of LYZ, without affecting cell functionality and integrity, as suggested by the absence of extracellular NAG and by the increase of intracellular NAG, which was probably induced by bacterial stimuli (Kelly & Carchman, 1987).

The results of this study show that low levels of tylosin could have a significant influence on immune and inflammatory molecules expression and concentrations as suggested by the presence of these activities at sub-optimal therapeutical levels and by the absence of any linear relationship among these parameters, tylosin concentrations and bacteria counts.

Conclusions

Successful mastitis cure is the result of achieving the optimal efficiency of both innate immune defences and

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therapeutic activity, by means of killing bacteria without eliciting an excessive inflammatory response. Therefore, antimicrobials for mastitis therapy should be selected not only on the basis of bacterial sensitivity, but also for their positive interaction with the innate immune response of the mammary gland. This study showed that an in-vitro model based on *Staph. aureus* challenge on MGEC could be helpful in assessing the interactions between invading bacteria and cells, intracellular/extracellular activity of antimicrobials and the influence of these latter on epithelial cell immune and inflammatory response.

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