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The Effects of Immunostimulants (Zinc, Levamisole, Vitamin AD$_3$E) Use Together With Enterotoxaemia Vaccine on Immunoglobulins in Sheep

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ABSTRACT

Objective: In this study, LMS, zinc and vitamin AD$_3$E were used with enterotoxaemia vaccine in sheep, in order to investigate their immunostimulant activities and also to make a comparison among them regarding their immunostimulatory properties.

Material and Methods: In the research 40 sheep were divided into 4 groups, each group consisted of 10 sheep. Group I only vaccinated against enterotoxaemia. In groups II, III and IV besides enterotoxaemia vaccine, LMS, zinc and vitamin AD$_3$E were used, respectively. Blood samples were taken on days 0 and 35 of the experiment. Serum levels of immunoglobulins (IgM, IgG, IgA and IgE) were determined using ELISA kits specific to sheep immunoglobulins. Hematological parameters and serum zinc levels were also determined.

Results: Levamisole-treated group showed a significant increase in serum level of IgM, and zinc-treated group demonstrated a significant increase in serum IgG level ($P<0.05$). In day 35, serum IgM level was the highest in LMS group (group II) based on its levels in groups III and IV ($P<0.05$) and also IgG level was highest in group II when compared to its levels in groups III and IV ($P<0.01$). In group II, statistically significant decrease in total WBC and lymphocyte counts and statistically significant increase in thrombocyte counts were determined on day 35 ($P<0.05$). In group III, statistically significant decrease in total WBC, neutrophil counts and hematocrit values were detected ($P<0.05$), although, these changes were within physiologically normal limits.

Conclusion: In conclusion, LMS stimulated the increase in IgM levels and zinc stimulated the increase in IgG levels without inducing adverse effect and the increase in antibody production resulted in the enhancement of humoral immune response to enterotoxamia vaccine. The use of LMS and zinc as immunostimulant together with vaccination is recommended.

Keywords: *Clostridium perfringens, Enterotoxaemia, Vaccination, Immunoglobulins, Immunostimulants*

INTRODUCTION

Sheep are economically multi-purpose animals which are reared for meat, milk, wool and hides. Pathogenic bacteria have adverse effects on the sheep health and without suitable protective measures bacterial diseases can become serious problems (Anonymous, 2015; Ramana et al., 2015).

The *Clostridium* genus includes more than 120 species that form a various group of rod shaped, Gram positive bacteria which can form spores. *Clostridium perfringens* (*C. perfringens*) may be the most widely occurring pathogenic bacterium and is certainly the most important cause of clostridial enteric disease in domestic animals. *C. perfringens*

produces a highly lethal disease in sheep, goats and other animal species, most of which are generically called enterotoxaemias (Uzal, 2004; Uzal and Songer, 2008). *C. perfringens* is classified into types A, B, C, D and E based on their ability to produce four types of lethal toxins, namely alpha, beta, epsilon and iota toxins (Songer, 1996; Shrestha et al., 2016). Under normal conditions, they do not harm the host and even may induce immune response to the toxins made by them. When large quantities of undigested carbohydrate and protein passed into the small intestine, as in cases of sudden change of high roughage rations to high grain rations or when sheep overeat on rich concentrated diets, the organisms proliferate rapidly and produce potent toxins resulting in enterotoxaemia (de la Rosa et al.,1997; Gershwin, 1999; Uzal and Songer, 2008).

Treatment of sheep showing clinical signs of enterotoxemia is seldom beneficial because of the rapid course of the disease and the animal may die without clinical signs (Niilo, 1988). Generally, enterotoxamia is prevented by vaccination along with good management. Many commercial vaccines are generally available for this purpose, but polyvalent clostridial vaccines are preferred (Altuğ et al., 2013; Altuğ and Muz, 2017).

Immune system is the only system among all body systems equipped to protect the body from microbial or parasitic infection (Quinn et al., 2011). The immune responses are mainly performed by immunoglobulins (Igs) or antibodies (Abs) (Mix et al., 2006). There are five isotypes of Igs: IgG, IgM, IgA, IgD and IgE. They have a common basic structure consisting of four polypeptide chains joined together by disulfide bonds (Gershwin, 2008). Only IgM and IgG activate complement which is essential in innate defense against bacterial and other infections and only IgE binds to mast cells, basophils and eosinophils which is important in allergies and protection against parasites (Tizard, 2004; Callahan and Yates, 2014). IgA is mainly found in the secretions washing the mucosal surfaces and relatively small amounts may be found within the circulation (Day and Schultz, 2011). IgD mainly found on the surface of B lymphocytes and its function is not well understood (Tizard, 2004; Schroeder and Cavacini, 2010).

Sometimes a vaccination program may not be successful and does not produce sufficient immunity against a specific disease so it is important to potentiate immunity by some agents called immunostimulants or immunopotentiators (Undiandeye et al., 2014) which are biological or synthetic agents enhancing both humoral and cell mediated defense mechanisms through activation of macrophages, neutrophils, natural killer cells, T lymphocytes and production of lymphokines. In veterinary medicine, immunostimulant agents are most commonly used for treatment and prevention of infectious diseases and also used to improve immunosuppression caused by nutritional deficiency, physiological and environmental stress (Mulero et al., 1998; Ali et al., 2012; Syed Ali Fathima et al., 2012; Ramana et al., 2015).

Levamisole (LMS) is a derivative of imidazothiazole and exerts toxic effect on a broad range of gastrointestinal and systemic nematodes in man and animals by inducing spastic paralysis and persistent muscular contraction in these nematodes. It also stimulates Ab formation against different antigens and enhances cell mediated immunity by stimulating T lymphocyte differentiation, it also enhances dendritic cell maturation, restores functions of monocytes and macrophages, and increases mobility and chemotaxis of neutrophils (Dörücü et al., 2005; Ali et al., 2012; Chandy et al., 2016). Zinc (Zn) is an important trace element in all organisms and plays role as a cofactor in more than 300 enzymes having influence on different organ functions. Zn is necessary for normal growth and function of immune system. It influences both innate and acquired immunity (Guzman-Rivero et al., 2014). Vitamins have important influence on immune responses and impaired immunity can be improved by providing moderate quantities of them (Chandra, 1997). Vitamin A is necessary for normal epithelial cells (Abdelrahman and Al-karablieh, 2002). It stimulates cellular immunity and Ab production (Villamor and Fawzi, 2005). Vitamin D has effects on regulation of immunity (Priyantha et al., 2012). Vitamin E which is an antioxidant can eliminate free radicals and prevent peroxidation of polyunsaturated fatty acids, thus protecting the immune cells and augments phagocytosis (Tengerdy, 1990; Sikka et al., 2002).

In this research, three immunostimulant agents (LMS, Zn and vitamin AD$_3$E) were used with enterotoxaemia vaccine to investigate their effects on the production of Igs in vaccinated sheep and maintaining immunity for longer time. Furthermore, immunostimulatory effects of these agents were also aimed to compare.

MATERIALS and METHODS

Materials

In this study 40 healthy female animals were selected from a flock of sheep in the region of Van. All routine clinical examinations were performed on the animals according to the methods described in clinical examination procedures (Başoğlu, 1998). The animals have been treated for internal and external parasites 30 days before commencing the experiment in order to be free from parasitic agents. These healthy sheep were divided into 4 groups and each group was composed of 10 animals.

On days 0 and 35 of the research 6ml of blood was taken from jugular vein of each animal, 2.5 ml of which was stored in EDTA-containing tubes for hematological analysis and the remaining blood was stored in test tube containing clot activator for measuring serum levels of Igs and Zn. All serum and plasma samples in eppendorf tubes were preserved in the freezer (-20 ºC).

After all blood samples were taken on day 0, enterotoxamia vaccine which was available in the market and approved by the Ministry of Food, Agriculture and Livestock (Republic of Turkey) was administered to all sheep in the four groups as the following:

Group I. Only enterotoxaemia vaccine (Ultrabac® 8 -Pfizer) (1 ml SC) was used.

Group II. In this group, enterotoxaemia vaccine (Ultrabac® 8 -Pfizer) (1 ml SC) and levamisole tablet (5 mg/kg/bw PO) (Zelensin®, Sanovel) were used.

Group III. In this group, enterotoxaemia vaccine (Ultrabac® 8-Pfizer) (1 ml SC) and zinc tablet (30 mg/50/kg/bw PO) (ZINCO® 30 mg tab. BERKO) were used.

Group IV. In this group, enterotoxaemia vaccine (Ultrabac® 8 -Pfizer) (1 ml SC) and vitamin AD_3E (Vit A 500.000 IU, Vit D 75.000 IU, Vit E 50 mg - IM) (ADEMİN®-CEVA-DİF) were injected.

On day 7, another dose of levamisole (5 mg/kg/bw PO) was given to all sheep in group II. On day 21, the second dose of enterotoxaemia vaccine was administered to all animals in the four groups.

Methods

Clinical Examinations

During the experiment general physical examination was performed for all sheep and heart rate, respiratory rate, body temperature, mucous membrane and skin were regularly checked for any sign of disease.

Hematological Analysis

Blood samples were analyzed by Veterinary Hematology Analyzer (Abacus - Junior Vet5®). Blood parameters including Red Blood Cells (RBCs), Hemoglobin (HGB), Hematocrit (HCT), White Blood Cells (WBCs), Lymphocytes (LYM), Monocytes (MONO), Neutrophils (NEUT), Eosinophils (EOS) and Platelets (PLT) were measured on days 0 and 35 of the research.

Biochemical Analysis

Measurement of Ig levels: ELISA test kits (CUSABIO®, China) were used for measuring serum levels of Ig isotypes (IgM, IgG, IgA and IgE) in the obtained serum samples as explained in the procedure of ELISA kits (CUSABIO® BIOTECH CO., Ltd.) in ELISA reader (ELISA reader®- DAS). A software program (Curve Expert 1.3) was used to make a standard curve. By marking the optical density of the samples on the standard curve, Ig levels were determined.

Measurement of Zn levels: Zn levels were determined in the serum samples by Atomic Absorption Spectrometer (AAS) (Thermo®).

Statistical Analysis

Descriptive statistics such as mean and standard deviation values have been expressed for comparison among groups of the experimental sheep. Kruskal Wallis test was used to make comparison of the groups in terms of these features. In addition, Wilcoxon test was utilized in comparison of before and after values in each group. In the calculations level of statistical significance was taken as 5% and SPSS statistical package program was implemented in these calculations.

RESULTS

Clinical Findings

Vital signs including heart rate, respiratory rate, body temperature, and mucous membrane and skin conditions were evaluated. From day 0 to day 35, no animal has lost and no abnormal signs were recorded.

Laboratory Findings: Hematological parameters and serum levels of Igs and Zn obtained from all groups of experimental sheep were given in tables 1, 2 and 3, respectively.

Table 1. Hematological findings in the experimental sheep on days 0 and 35

Parameters	Day of Sampling	Group I [Only vac.] (n=10)	Group II [Vac. + LMS] (n=10)	Group III [Vac. + Zn] (n=10)	Group IV [Vac.+AD3E] (n=10)
WBC (10^9/L)	0	10.20±0.50	10.60± 0.72	10.18± 0.75	10.25 ±0.98
	35	9.53±1.28	8.18± 0.91*	7.36± 0.34 *	8.31 ±0.74
LYM (10^9/L)	0	2.51± 0.43	4.06± 0.23	3.68 ±0.52	3.26± 0.44
	35	3.46± 0.63	3.18± 0.14 *	3.43± 0.26	3.86± 0.14
MONO (10^9/L)	0	1.77± 0.17	1.39± 0.16	1.56± 0.16	1.60± 0.18
	35	1.53± 0.38	1.13± 0.20	1.21± 0.03	1.23± 0.06
NEUT (10^9/L)	0	6.33± 0.66	5.06± 0.53	4.16± 0.18	4.68± 0.30
	35	4.36± 0.44	3.80± 0.61	2.66± 0.18 *	3.20± 0.69
EOS (10^9/L)	0	0.20± 0.05	0.08± 0.03	0.10± 0.06	0.05± 0.02
	35	0.10± 0.01	0.06± 0.02	0.05± 0.02	0.05± 0.03
RBC (10^{12}/L)	0	9.25± 0.66	9.31± 0.53	9.41± 0.47	7.98± 0.61
	35	7.95±0.52	8.36± 0.57	8.38± 0.24	7.94± 0.44
HGB (g/dl)	0	9.33± 1.15	10.33± 1.63	10.17± 1.17	8.67± 1.75
	35	8.67± 1.15	9.33± 2.16	9.17± .75	8.83± 1.33
HCT (%)	0	28.00± 4.36	31.17± 4.79	31.17± 3.25	26.00± 4.47
	35	24.67± 3.79	27.17± 5.53	27.00± 1.79 *	26.00± 4.05
PLT (10^9/L)	0	301.00± 51.86	309.50± 29.29	424.33± 92.57	617.50±69.13
	35	392.67±93.29	494.83± 64.13*	454.67± 70.42	756.42± 55.24

* is statistically significant according to the "Day 0" of the same group (P<0.05).

Table 2. Serum levels of Igs in the experimental sheep on days 0 and 35

Parameters	Day of Sampling	Group I [Only vac.] (n=10)	Group II [Vac. + LMS] (n=10)	Group III [Vac. + Zn] (n=10)	Group IV [Vac.+AD3E] (n=10)
IgM (µg/ml)	0	44.21±9.69	52.24±2.09	50.79±5.19	40.01±1.58
	35	55.22±7.64	84.89±14.22*[a]	58.58±4.30[a]	57.01±8.41[a]
IgG (µg/ml)	0	46.90±11.71	64.07±8.24	39.45±5.49	33.63±4.90
	35	50.69±12.08	82.26±11.59[b]	57.75±3.79*[b]	36.61±4.77[b]
IgA (µg/ml)	0	17.62±2.34	16.79±2.69	22.23±1.67	27.70±4.11
	35	42.16±9.54	28.21±4.11	25.80±2.91	31.64±3.26
IgE (ng/ml)	0	438.37±125.58	628.38±189.11	726.11±246.09	447.45±171.12
	35	350.78±137.23	590.03±137.16	508.58±336.54	491.17±139.30

[a,b] are statistically significant among different groups in the same line. [a](P<0.05), [b](P<0.01)

Table 3. Serum levels of Zn in the experimental sheep on days 0 and 35

Parameters	Day of Sampling	Group I [Only vac.] (n=10)	Group II [Vac. + LMS] (n=10)	Group III [Vac. + Zn] (n=10)	Group IV [Vac.+AD3E] (n=10)
Zinc levels (mg/L)	0	0.83±0.07	0.66± 0.18	0.59± 0.31	0.57± 0.11
	35	0.71± 0.13	0.49± 0.23	0.64± 0.10	0.71± 0.18

Hematological Findings: According to the WBC count on day 0 (Table1), statistically significant decrease in WBC levels were discovered in groups II and III (P<0.05). WBC levels were also decreased in groups I and IV but these changes had no statistical significance.

A statistically significant decrease in LYM count was observed in group II on day 35 of the experiment (P<0.05). No statistically significant changes were recorded in other three groups. According to the NEUT value on day 0, a statistically significant decrease was shown only in group III on day 35 (P<0.05). None of other groups showed significant changes.

No statistically significant changes were observed in MONO, EOS and RBC counts, and in HGB level in experimental groups. On day 35, a statistically significant decrease was recorded in percentage value of HCT in group III (P<0.05). In other groups, no statistically significant change in its value was shown. According to PLT counts on day 0, a statistically significant increase was only recorded in group II (P<0.05). PLT were also increased in groups I, III and IV but none of these changes were significant statistically.

Biochemical Findings

According to IgM level in group II on day 0, a statistically significant increase occurred in its level on day 35 (P<0.05). IgM levels were also increased in other three groups on day 35 but statistically not considered significant. Statistically significant variation was found among groups II, III and IV on day 35 (P<0.05).

On the basis of IgG level on day 0, a statistically significant increase in its level was found in group III, on day 35 (P<0.05). Non-significant elevation in IgG levels occurred in groups I, II and IV (P>0.05).

According to IgA values on day 0, its levels were increased in all groups on day 35 of the experiment, although the changes were not considered significant statistically. No significant change occurred in IgE values in any group on day 35 based on day 0 values. Based on serum levels of Zn on day 0, its values were changed in all groups of sheep on day 35. Its levels were decreased in groups I and II but increased in groups III and IV. None of them were statistically significant.

DISCUSSION AND CONCLUSION

It has been observed that, vaccinated animals still suffer from the disease against which vaccination has been done, for this reason, immunostimulatory agents were employed together with vaccination to ensure a more potent and lasting immunity (Undiandeye et al., 2014).

In this research, LMS, Zn and vitamin AD$_3$E were used with enterotoxamia vaccine in order to investigate their immunostimulatory effects on immune responses via determining the Ig levels and also comparing their immunostimulatory properties.

No statistically significant variations were found in the blood parameters, Igs or Zn levels in group I, on day35 (Tables1, 2 and 3).

In group II, total WBC and LYM counts have significantly decreased (P<0.05) but were within normal range. This result was in contrast to those of Mohri et al. (2004) and Pekmezci and Cakiroglu (2009) reported that no significant changes were found in total WBCs and LYM. In another scientific work, Aly et al. (2010) discovered that WBC count was not increased significantly in LMS-treated group whereas significant increase occurred in the group which received LMS with the vaccine.

PLT count has risen significantly (P<0.05) in this group but has remained within normal limit.

Non-significant decreases were recorded in number of MONO, NEUT and EOS. These results agreed with those of Bisalla et al. (2009) who used LMS in sheep with acute experimental *Trypanosoma congolense* infection and found that no significant change has occurred in absolute values of MONO, NEUT and EOS in all sheep. But they were contrary to the results of Krakowski et al. (1999) that revealed a significant increase in phagocytic activity of neutrophils and also contrary to the results of Mohri et al. (2004) which found that LMS has caused a significant increase in NEUT and MONO. In another study Pekmezci and Cakiroglu (2009) found that LMS has caused a significant increase in MONO. This finding was in contrast to the present research. Value of EOS in the group II might indicate the safety of LMS for immunological purposes without inducing allergic reactions in the animals.

In this study, the non-significant decrease was discovered in each of RBC, HGB and HCT in group II but they were within normal limits. These results were similar to those found by Pekmezci and Cakiroglu (2009) and Aly et al. (2010). Non-significant change in HCT was also found by Mohri et al. (2004). This result was similar to HCT result in the present research. Mulero et al. (1998) administered different doses of LMS to teleost

gilthead seabream and found that HCT values were significantly decreased in LMS-treated groups of fishes, 0 and 5 weeks after the last use of LMS.

The main Ig occurring during a primary immune response is IgM and it also appears in a secondary response but not as high as IgG (Tizard, 2004; Mix et al., 2006; Schroeder and Cavacini, 2010). In group II, IgM level was significantly increased (Table 2). This means a better and lasting immunity. This might be due to combined effect of both enterotoxaemia vaccine and LMS. LMS alone might not have this immune modulating effect as Undiandeye et al. (2014) revealed that LMS alone did not induce the production of immune cells. Whereas, Shah et al. (2011) discovered that responsiveness of T and B lymphocytes was enhanced when LMS used alone. Different results might be related to various methods of using LMS. Shah et al. (2011) used LMS for a long course of 15, 30 and 45 days in three groups of rat in order to check whether it can maintain immunity in chronic cases. Whereas Undiandeye et al. (2014) used LMS for 4 days which was a short course when compared to period of 15, 30 and 45 days.

In group II, LMS stimulated the production of IgG and IgA and their levels were elevated but statistically not considered significant. Similar results were found by Krakowski et al. (1999). Maĭchuk and Mikuli (1981) discovered that LMS has increased local IgA secretion in lacrimal fluid in normal and sick rabbits but IgG secretion was lower in sick rabbits than in normal rabbits. Serum levels of IgE were decreased on day 35. This confirmed that neither enterotoxamia vaccine nor LMS has stimulated allergic reactions in group II. No significant change has been recorded serum Zn levels in group II.

In group III, a significant decrease in total WBC and NEUT counts ($P<0.01$) was recorded in group III but these changes were within normal limit. Anon-significant decrease in LYM, MONO and EOS occurred in group III. The reverse of these findings were shown by Eze et al. (2015) that found a significant increase in WBC count and Ab titer as a result of Zn administration. In two separate researches, conducted by Bao et al. (2008) and Sobhanirad and Naserian (2012), Zn supplementation has not made significant differences in total WBCs, LYM, MONO, granulocytes and PLT. In another scientific work by El Hendy et al. (2001), experimentally induced Zn deficiency in growing rats has caused a significant increase in total WBCs.

Also in this group, a significant decrease in HCT values and non-significant decreases in RBCs and HGB concentration were observed. These findings were contrary to those of Bao et al. (2008) and Sobhanirad and Naserian (2012) that found Zn supplementation caused a significant increase in RBC, HGB and HCT values.

Group III demonstrated increased levels of IgM, IgG and IgA. A significant increase only occurred in IgG concentrations. Increased concentrations of IgM were not considered significant. This might be due to the fact that IgM soon appears in acute immune response and its level might decrease after 35 days (duration of this research). Whereas IgG is a dominant Ig appears in secondary immune response (Tizard, 2004) which means it remains high for a longer time than IgM do. These results were similar to those of Guzman-Rivero et al. (2014) that reported Zn supplementation to tuberculosis patients has increased plasma levels of IgM, IgG and IgA and also to those of Prasad and Kundu (1995) that observed high IgG and IgM responses in calves fed Zn-supplemented milk. But contrary to the results of Habib et al. (2015) that discovered Zn supplementation had no influence on immunogenicity of oral poliovirus vaccine. In this group, IgE levels were decreased indicating that Zn had no induced allergic reactions.

Increased serum Zn levels were recorded in sheep in group III (Table 3). Although statistically was not significant, but when compared to the Zn levels in other experimental groups, the most increased level was found in this group. This was due to the effect of orally administered Zn, similar result was found by Habib et al. (2015), Prasad and Kundu (1995) and Akbari Moghaddam Kakhki et al. (2016).

In group IV, no significant changes were observed in total and differential WBC counts. Total WBC, MONO and NEUT counts decreased and LYM count slightly elevated. Vitamin AD_3E might have exerted more effect on humoral immunity than on cell-mediated immunity. This can be evidenced by increases in serum Ig concentrations and also in LYM count.

Generally, the serum levels of all Igs were elevated in this group, although these changes were not significant.

Kizil and Gul (2008) administered vitamin AD_3E with Foot and Mouth Disease (FMD) vaccine to a

group of cattle. The result was a good protection against FMD. This result might explain the anti-stress property of vitamin AD3E which has minimized the negative effects of the vaccination. Naimi et al. (2014) studied the effect of vitamin AD3E on performance and humoral immune response in broiler chicks and concluded that vitamin AD3E has enhanced humoral immunity probably due to the role of vitamin E which has increased lymphocyte activity.

Syed Ali Fathima et al. (2012) reported that vitamin A has enhanced the immune capacity and general resistant against the pathogens in fish. Earlier, Thompson et al. (1995) observed that vitamin A had a small immunostimulatory effect on total Igs in rainbow trout when used in their diet. Also Fan et al. (2015) investigated the influence of dietary vitamin A supplement levels in chicks and found that vitamin A supplementation significantly elevated the level of IgA in the bronchoalveolar lavage fluid and serum.

Significant differences were found in IgM and IgG levels among groups II, III and IV on day 35. IgM and IgG levels in Group II were higher when compared with their levels in groups III and IV (Table 2). The different levels of IgM and IgG in each group reflected the degree of immune stimulating activity of the agents used in this research. Based on IgM and IgG results, LMS was the most potent immunostimulant agent followed by Zn and, to a lesser extent, vitamin AD3E.

In group IV a slight rise was noticed in Zn level (Table 3). This result might explain the effect of AD3E on serum Zn level which was very little. The opposite of this result was obtained by Sikka and Lal (2006) which found a significant elevation in serum Zn level in calves born to vitamin AD3E-supplemented buffalos.

Anugu et al. (2013) discovered that supplemented vitamin E has increased serum level of IgG following vaccination in late pregnancy. Samanta et al. (2006) found out that, vitamin E supplementation in the diet of calves might enhance the humoral immunity of them against *Pasteurella multocida* whole cell antigen.

In conclusion, it was found that LMS and Zn augmented the immune response to enterotoxaemia vaccine especially during immune suppression and immunostimulants are beneficial to potentiate immune response to vaccines since sometimes vaccination alone may not properly stimulate immunity against a pathogen; Thus it is recommended to use these immunostimulant agents with vaccines to induce better immunity lasting for a longer time. Finally, these agents are safe to use without inducing allergic reactions and also they improve overall health conditions of the animals.

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