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Original article

Serum amyloid A and TNFα in serum and milk during experimental endotoxin mastitis

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Abstract – A cross-over study was conducted to investigate the effect of intramammarily infused lipopolysaccharide (LPS) on the acute phase reaction in early (EL) and in late (LL) lactation. Nine cows received intramammary injections of 100 μg of *Escherichia coli* 0111:B4 LPS during EL and LL. The severity of each cows systemic and local signs and change in milk appearance were recorded and scored throughout the experiment. Systemic and local signs were found to be more serious in EL cows. Tumor necrosis factor α (TNFα) was detected in milk but not in serum. Serum amyloid A (SAA) concentrations increased both in serum and in milk. The milk TNFα concentrations peaked at 8 h post-challenge (PC). SAA concentrations started to increase at 8 h PC, and peak concentrations were seen at 32 and 48 h PC in milk and serum, respectively. The milk TNFα and SAA seemed to be correlated, being on average higher in EL. Serum SAA concentration was not correlated with milk TNFα or SAA, nor with the severity of local or systemic signs, but was correlated with changes in milk appearance.

LPS / mastitis / dairy cow / SAA / TNFα

1. INTRODUCTION

Mastitis caused by *Escherichia coli* is a common disease in lactating dairy cows. Studies on spontaneous or experimental *E. coli* mastitis have shown that affected cows can be divided into mild or severe responders, based on their clinical signs and disease outcome [8, 26, 34, 35]. Periparturient cows and those in early lactation most frequently show severe clinical signs and even fatal outcome [4, 7, 18]. The observed systemic and local clinical reactions result from the acute phase reaction (APR), which is the response of the host to any tissue injury caused by trauma or inflammation [32].

During *E. coli* mastitis, lipopolysaccharide (LPS) released from bacteria is thought to be the initiating factor of APR [4, 21]. LPS stimulates mammary monocytes and macrophages to produce pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukins 1, 6 and 8 (IL-1, IL-6, IL-8) [15, 32]. These in turn act both locally and systemically, attracting polymorphonuclear neutrophils (PMN) from the

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circulation to the infection site and inducing the production of acute phase proteins (APP) in the liver.

The role of TNFα in the pathogenesis of *E. coli* mastitis has been studied widely. TNFα seems to have a critical role in initiating host response e.g. by possibly inducing the production of IL-1, IL-6 and IL-8 [22, 33]. TNFα is not a potential chemoattractant but can prime neutrophils to express adhesion molecules and thus support PMN migration [15]. The cytokine IL-8 is a potent chemoattractant for neutrophils and can be released without TNFα at the early stages of LPS-induced inflammation [15, 23]. On the contrary, at a later stage of inflammation the effect of TNFα may turn deleterious to the host if the production of TNFα increases to high levels [15]. Several authors have found correlations between the severity of response and concentrations of TNFα in the blood or milk [3, 8, 9, 20]; in other studies, no direct correlation has been seen [10, 19]. In LPS mastitis models, TNFα was detected in the plasma and milk of early lactating cows [3, 10], but not in cows in midlactation [22]. In the latter study, the amount of LPS used for the induction of mastitis was much lower than in the first two studies.

Serum amyloid A (SAA) is a sensitive APP in cattle [1, 8, 11, 13, 24]. SAA has been shown to reflect the severity of mastitis; cows with clinical mastitis had almost 100-fold levels of SAA in serum and milk compared with healthy cows [5]. SAA is mainly produced in the liver but is also produced extrahepatically by macrophages, endothelial cells and smooth-muscle cells [5, 12, 17, 30]. The effects of SAA may differ locally and systemically and may be concentration-dependent [30]. Local and systemic production of SAA may also be governed by different control mechanisms [5].

Differences in the production, function and kinetics of cytokines and APP may explain the varying local and systemic signs of individual cows in *E. coli* mastitis [3, 8, 18, 20, 25]. We used an experimental LPS model to study local and systemic acute phase responses of dairy cows in different lactation stages. Clinical findings of these cows have previously been published in detail [16]. Our aims were to investigate (1) the kinetics and concentrations of TNFα and SAA in serum and milk, (2) the connection between both TNFα, SAA and clinical signs and (3) the effect of lactation stage on the TNFα and SAA concentrations in LPS induced mastitis.

2. MATERIALS AND METHODS

2.1. Animals and experimental protocol

Nine Finnish Ayrshire cows were used in the experiment. Two of the cows were primiparous and seven multiparous (2nd–4th lactation). All cows were clinically healthy and their udder quarters were free from mastitis pathogens and had a low milk SCC (< 150 000 cells/mL) before the experiment. The cows were housed in a stanchion barn and fed freely with good quality silage and hay. Concentrate was given twice daily. The cows were milked two times a day, at 7.00 am and at 5.00 pm.

The cows were challenged with LPS twice; once in the early lactation (EL) period 6 to 15 days after parturition, and once in the late lactation (LL) period 137 to 77 days before the next parturition. A cross-over design was used and the cows were randomly allocated into two subgroups; one group was challenged first in the EL period and the other group in the LL period. The cows in the latter group thus calved before the second challenge. The LPS challenge was done after the morning milking into one hind quarter with 100 μg *Escherichia coli* 0111:B4 lipopolysaccharide B (Bacto©, Difco Laboratories, Inc., Detroit, USA) diluted into 5 mL of sterile isotonic NaCl. The same quarter was used at both challenges. During the experiment the cows did not receive any treatments.

The ethics committee of the Faculty of Veterinary Medicine, Helsinki, Finland, approved the study protocol.

2.2. Clinical examination and sampling

Systemic and local signs were recorded throughout the experiment at 0, 2, 4, 8, 12, 24, 32, 48 and 72 h post-challenge (PC). The cows' systemic and local clinical signs and milk appearance were assessed using a three-point scoring system, including half numbers [26]. Score 1 was categorized as normal, and 3 as a severe reaction, i.e. with a rectal temperature > 40.5 ºC, anorexia and depression among the systemic signs, severe swelling and pain in the udder among local signs, and milk appearance changing to serous or clotty.

Milk samples were collected from the challenged quarters of each cow at 0, 4, 8, 12, 24, 32, 48 and 72 h PC. Blood samples were collected from the jugular vein in plain tubes at the same time points as the milk samples, and serum was separated by centrifuging within 24 hours. Milk samples were stored frozen at –21 ºC and serum samples at –70 ºC.

2.3. Analytical methods

2.3.1. TNFα analysis

The enzyme linked immunosorbent assay (ELISA) used in this study was modified from Ellis et al. [6]. The following adjustments were made for quantification of TNFα in serum. Serum samples were diluted 1:2 in tris-buffered saline (TBS: 0.05 M Tris, 0.15 M NaCl, pH 7.6) containing 0.05% tween and 0.5% gelatin (Sigma-Aldrich Co., St. Louis, USA) (TBS-T-g), and duplicates of the samples were added to two coated plates and incubated overnight at 4 °C. A 2-fold dilution of recombinant bovine TNFα (Ciba-Geigy, Basel, Switzerland) in 50% TBS-T-g and 50% FCS was used as the standard starting at 62.5 ng/mL. A high and a low positive control were added in a ratio of 1:2. A mixture of 50% TBS-T-g and FCS was used as a negative control.

The same ELISA was slightly modified to analyse TNFα from the milk samples. Before the TNFα analysis, milk samples were centrifuged at 25 000 *g* for 40 min at 4 °C. The fat layer was removed and the supernatant was collected. The supernatants were then centrifuged again at 2 000 *g* for 20 min at 4 °C to remove the final remnants of fat. The positive and negative controls for milk samples were treated in a similar way. The milk supernatants were diluted from 1:4 to 1:512 (2-fold dilution) in TBS-T-g to find the most appropriate dilution. The diluted samples were tested in triplicate. The diluted samples were added to coated plates and were incubated overnight at 4 °C. A 2-fold dilution of recombinant bovine TNFα in TBS-T-g with 6.25% milk supernatant was used as a standard starting at 62.5 ng/mL (the bovine rTNFα stock was 3.125 µg/mL and kept in TBT-g with 10% heparin stabilized bovine plasma). A high positive control was added in dilutions of 1:64 and 1:128, and a low positive control in a dilution of 1:8. TBS-T-g with 6.25% milk supernatant was used as a negative control. The following day, the procedure was continued as described above.

The inter-assay (between days) and intra-plate coefficients of variation (CV) for the serum TNFα-ELISA were below 14.4% and 14.8%, respectively, and for the milk TNFα-ELISA were as follows: the low control (5.8 ng/mL diluted 1:8) 9.8% and 6.7%, and the high control (59.5 ng/mL diluted 1:64) 6.6% and 9.1%.

The detection limit of the ELISA was 0.5 ng/mL for the serum and around 1.0 ng/mL for the milk.

2.3.2. SAA analysis

The concentration of SAA in serum and milk was determined using ELISA (Tridelta Development, Wicklow, Ireland) as described by Eckersall et al. [5]. For high SAA concentrations, the samples were diluted as

necessary. The detection level was > 0.3 μg/mL for both serum and milk samples, and the upper limit for the analysis was < 750 μg/mL. The inter-assay and intra-assay coefficients of variation for SAA analysis were < 10% and < 5%.

2.4. Statistical analysis

Repeated measures analysis of variance with stage and hour as within factors was used to test the effect of lactation stage on rectal temperature, TNFα in milk and SAA in serum and milk.

The model was as follows:

$$Y_{ijk} = \mu + \alpha_j + \beta_k + (\alpha\beta)_{jk} + \pi_i + (\pi\alpha)_{ij} + (\pi\beta)_{ik} + (\pi\alpha\beta)_{ijk}$$

where Y_{ijk} = value of a variable for ith cow at stage j in hour k, μ = grand mean, α_j = effect of stage, β_k = effect of hour, $(\alpha\beta)_{jk}$ = stage × hour interaction effect, π_i = effect of cow, $(\pi\alpha)_{ij}$, $(\pi\beta)_{ik}$ and $(\pi\alpha\beta)_{ijk}$ = cow × stage, cow × hour and cow × stage × hour interaction effects.

Mean squares corresponding to terms $(\pi\alpha)_{ij}$, $(\pi\beta)_{ik}$ and $(\pi\alpha\beta)_{ijk}$ were used as the denominators in F-ratios when testing the stage, hour and stage × hour effect, respectively.

Significance of hour and stage × hour effect were evaluated by Greenhouse-Geisser adjusted *P*-values. In case of a significant overall effect, successive hours were further contrasted. Significance was set at $P < 0.05$. The results are expressed as mean (± standard error).

3. RESULTS

3.1. Local and systemic signs

All cows developed clinical signs of mastitis after the LPS challenge. Clinical systemic signs were more severe in EL than in LL cows. Systemic signs peaked at 4 or 8 h PC and returned to normal at 32 h PC (Fig. 1). The rectal temperatures started to increase at 2 h PC; this increase was significantly ($P < 0.01$) more pronounced in EL than in LL between 2 and 4 h PC. The peak temperature occurred at 8 h PC, being 40.3 °C in EL and 40.1 °C in LL (data not shown). Severe or moderate local signs were present in the udder already at 2 h PC, peaking at 4 h PC (Fig. 1). The appearance of the milk changed later than the local signs (Fig. 1); milk turned serous and yellow on average at 8 h PC in EL and at 4 h PC in LL. More severe changes in milk appearance were seen in LL than in EL cows, but individual variation was considerable. The severity of systemic signs varied more than the severity of the local signs between the cows and lactation stages (Fig. 1).

3.2. Concentration of TNFα

No detectable levels of TNFα were found at any time point in the serum. The concentration of TNFα in the milk started to increase after the LPS infusion and peaked at 8 h PC (Fig. 2). The peak concentration was higher in EL than in LL, the respective means being 111.9 ng/mL and 33.9 ng/mL. The difference was not statistically significant due to the high individual variation.

3.3. Concentration of SAA

Intramammary LPS caused a significant ($P < 0.001$) increase in both serum and milk SAA concentrations (Fig. 2). Initial SAA values were 14.9 (2.1–67.1) μg/mL in serum and close to the lower detection limit (0.3 μg/mL) in milk. The concentrations of SAA started to increase after 8 h PC, peaking at 32 h PC in milk and at 48 h PC in serum. No significant differences were seen between SAA levels in EL and LL. However, in comparison with the pre-challenge values, SAA values in serum were significantly higher in EL after 24 h PC ($P < 0.001$) and in LL after 12 h PC ($P < 0.05$), and SAA values in milk were significantly higher

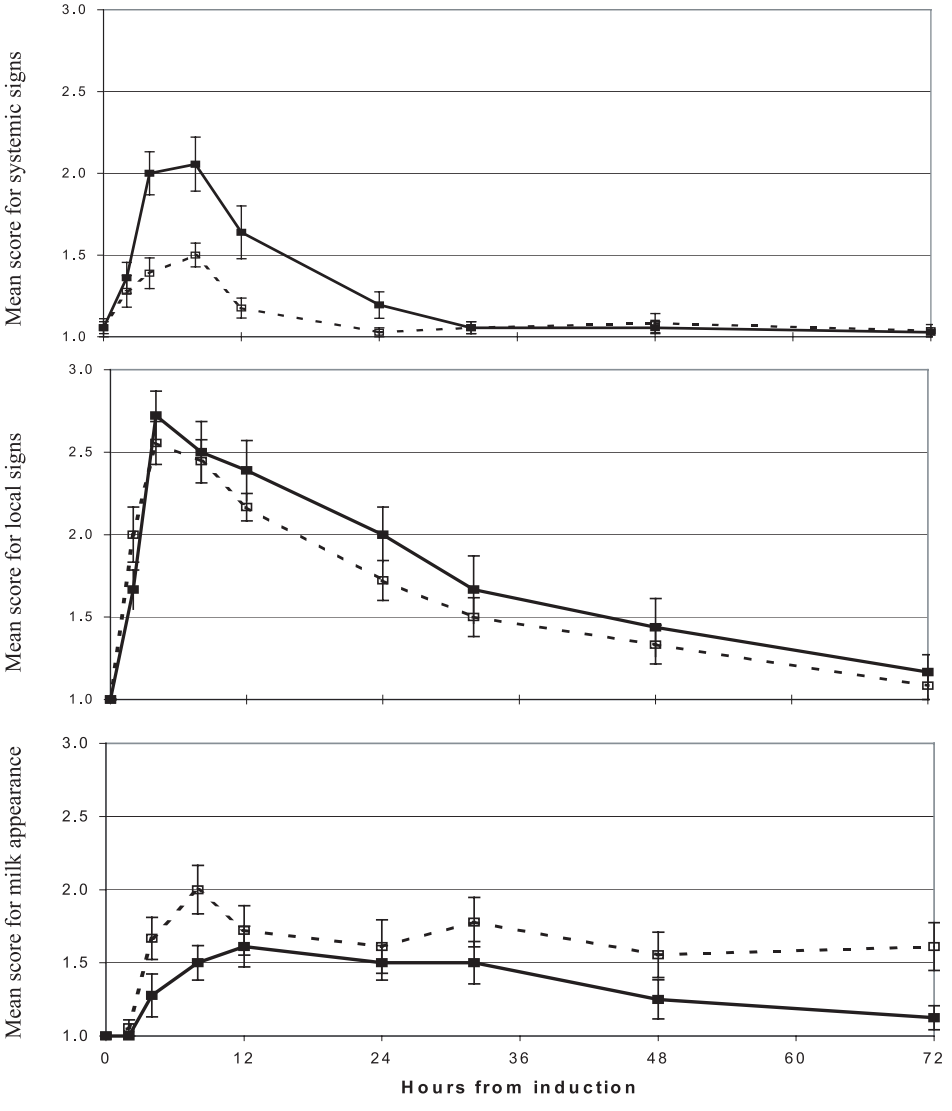

Figure 1. Change in cows' systemic and local signs and milk appearance after the induction of LPS mastitis. Data points represent the average (± SE) of nine cows. Values in early lactation are marked with solid symbols and lines, and those in late lactation with open symbols and dashed lines.

after 8 h PC in EL ($P < 0.05$) and after 24 h PC in LL ($P < 0.05$).

The individual time-concentration curves of SAA in serum were almost identical in EL and LL. The average peak concentration at 48 h PC was significantly ($P < 0.05$) higher in LL than in EL, 244.3 µg/mL and 193.5 µg/mL, respectively (Fig. 2).

In milk, the SAA concentrations were higher in EL than in LL (Fig. 2). Between 12 and 24 h PC, the concentrations increased significantly ($P < 0.05$) faster in EL than in

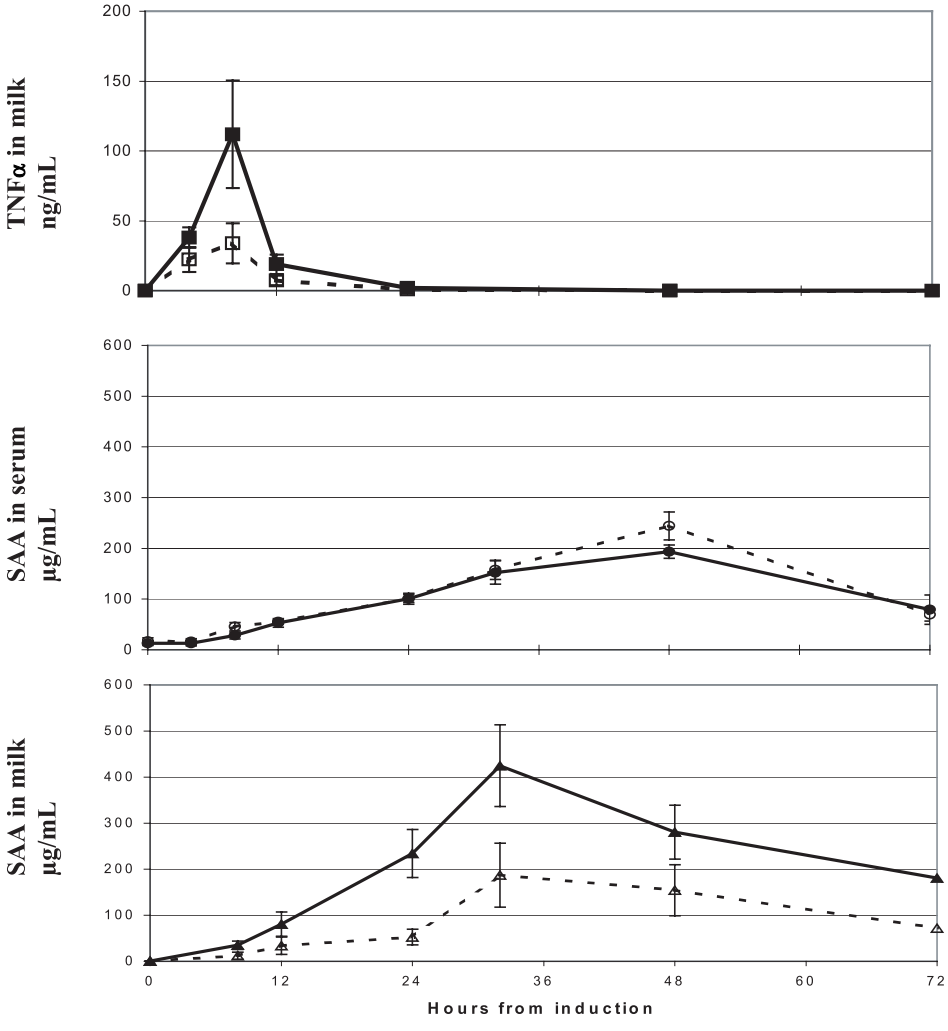

Figure 2. Change in TNFα (■) concentration in milk and SAA concentrations in serum (●) and milk (▲) in experimental LPS mastitis. Data points represent the average (± SE) of nine cows. Values in early lactation are marked with solid symbols and lines, and those in late lactation with open symbols and dashed lines.

LL, and a statistical difference was seen between the two lactation stages at 24 h ($P < 0.01$) and at 32 h PC ($P < 0.05$). The average peak concentrations were 424.6 µg/mL in EL and 185.8 µg/mL in LL at 32 h PC. Individual variation in the time-concentration curves was considerable.

4. DISCUSSION

Intramammary challenge with *E. coli* LPS induced a clear APR in all cows, observed as systemic and local signs and elevation of the inflammation parameters. Clinical signs and the changes of milk SCC

and NAGase have been described in detail in our previous article based on this study [16]. Clinical response varied from mild to moderate, but very severe cases were not seen, which is in accordance with previous LPS studies [3, 10].

TNFα was detected in milk but not in serum both in EL and LL. Increased TNFα concentrations in milk have been reported in earlier studies of experimental LPS [3, 10, 22] or *E. coli* mastitis [3, 8, 10, 28] and in spontaneous *E. coli* mastitis [9, 19, 20]. In serum, no TNFα has been found or the levels have been very low [3, 8, 10, 19, 20, 22, 28]. Differences in TNFα may be the result of variations in the study protocols and sample treatments between the studies. The trace amounts of TNFα in serum detected in some studies may have leaked from the mammary tissue to the circulation due to tissue damage.

SAA increased both in serum and in milk. The concentrations were higher than those found in spontaneous mastitis [5]. SAA peaked earlier in the milk than in the serum, but the concentrations of serum SAA did not follow those of milk (Fig. 2). This supports the idea that SAA must be produced and regulated both locally and systemically [5, 17]. In serum, the peak concentration of SAA was significantly higher in LL than in EL, but otherwise the concentrations were comparable, and individual differences were minimal. One reason for the lower serum SAA concentrations in EL compared with LL could be the compromised function of the hepatocytes during the peripartum period due to the fatty liver. While the concentration of SAA in the serum did not seem to be related to the systemic or local clinical signs, it was related to changes in milk appearance. Changes in milk appearance are partly caused by the loosened cell connections in the udder epithelia which allow the passage of proteins, enzymes, cells and trace elements between milk and blood. Some of the SAA detected in the serum may originate from the milk, because of this increased permeability.

TNFα not being found in serum may indicate that in cattle the systemic SAA production is not induced by TNFα but by some other cytokines, although the intradermal injection of recombinant bovine TNFα has been reported to cause some increase in the APP, namely haptoglobin [14, 33]. IL-6 increases in the serum after LPS stimulation or *E. coli* mastitis [19, 27], and it has been shown to be a potent inducer of hepatic production of SAA [2]. In milk, concentrations of TNFα and SAA seemed to be closely intertwined; cows with very low TNFα also had low SAA concentrations, and those with high TNFα had high SAA concentrations. The correlation between TNFα and SAA has also been observed in human patients with sepsis [30].

TNFα and SAA concentrations in milk and serum have been established to be correlated with the severity of the cows response [3, 5, 8, 9, 20, 30]. In our study, the severity of the response seemed to be associated with TNFα and SAA in milk; the average concentrations of TNFα and SAA were higher in EL (Fig. 2), in addition to more severe systemic and local signs (Fig. 1). However, no connection with the severity of the disease was seen in individual cows. This may be explained by the great individual variation of the cows' responses in general and the mild course of our LPS model.

As also reported in previous studies [3, 10, 15], TNFα concentration did not peak before the manifestation of clinical signs but was detected at the same time as the systemic signs and later than the local signs. Higher concentration of TNFα in milk in EL may not be connected with the severity of response but rather with differences in the immune system, including the cell populations in EL and LL. Sordillo et al. [29] found higher numbers of monocytes in EL cows and a greater ability of these cells to produce TNFα.

The role of SAA in the repair processes after tissue damage, detoxification of LPS [31] and down-regulation of the pro-inflammatory responses [30] is supported

by our findings that the increase in milk and serum SAA occurred after clinical signs decreased. At the time of the most severe signs, the SAA concentration had reached only one-fourth of the highest value. These early phase concentrations of SAA were similar to those found by Eckersall et al. [5] in clinical cases of mastitis. While the slow rise of the concentration of SAA in milk diminishes the prognostic value of milk SAA for assessing the severity of mastitis, SAA has a potential as a diagnostic indicator of mastitis [24].

In conclusion, intramammarily infused *E. coli* LPS caused an increase in milk TNFα and SAA and in serum SAA. Our results support previous suggestions that TNFα response to LPS is local and regulated at the level of the mammary gland [22], and that it is not responsible for the rise of body temperature or other systemic signs [10, 22]. However, milk TNFα and SAA seemed to be associated with each other and with clinical signs, and were on average higher in EL. Serum SAA concentration was not related to milk TNFα or SAA, nor was it related to the severity of local or systemic signs. Only slight differences were present in serum SAA between cows, and peak concentrations on average were higher in LL, which is also when the most severe changes in milk appearance were seen.

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