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Relationship of inflammatory cytokines with experimental Mannheimia haemolytica infection in ewes fed dam milk or cow milk after birth*)

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Summary

The purpose of this study was to determine the relationship between tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) cytokines and experimental Mannheimia haemolytica (M. haemolytica) infection in ewes that had been fed colostrum and dam milk or commercial cow milk after birth. Twenty one-year-old Chios ewes were fed colostrum and dam milk (n=10) or commercial cow milk (n=10) after birth while they were lambs. Feed and water were given ad libitum. Ewes were inoculated intratracheally with 1×10^9 M. haemolytica Type A1 in 2 ml of PBS. Blood samples were collected from the jugular vein once before bacterial inoculation and 3 times at day 1, 10 and 22 after bacterial inoculation from all animals. Antibiotic treatment was applied at the 22th day. Blood samples were also collected at day 7 of the treatment. Serum samples were stored in –70 °C until analysed. Serum TNF-α, IL-1β and IL-6 levels were assayed by enzyme-linked immunosorbent assay. Test results were analysed using a Student's t test between two groups and one way ANOVA between blood sampling days. The levels of TNF-α, IL-1β and IL-6 in ewes fed colostrum and dam milk tended to be higher than in ewes fed commercial cow milk; the differences, however, were not statistically significant. All levels insignificantly increased after bacterial inoculation and then gradually decreased. The results may indicate that the inflammatory response of ewes fed colostrum and dam milk after birth is relatively more potent than of ewes fed artificially. However, the differences observed in this study were not statistically significant.

Keywords: ewe, colostrum-deprived, cytokines, Mannheimia haemolytica

The alveolar macrophages are a major cell population in alveolar spaces and play an important role in the defense against respiratory tract infections by microorganisms. The activation and release of bacteriocidal substances from the alveolar macrophages are essential for fighting infection (23). The initial interaction of cell wall lipopolysaccharides of *Mannheimia haemolytica* (*M. heamolytica*), an endotoxin, with resident alveolar macrophages leads to production and release of soluble mediators such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), prostaglandin E_2, interferons and platelet-activating factor (6, 10). This is followed by recruitment of neutrophils, eruption of a cytokine-mediated inflammatory cascade, and neutrophil activation, resulting in the release of toxic oxygen radicals, proteases, and cytokines which participate in direct lung tissue injury (10).

Cytokines regulate local inflammatory reactions, have effects on phagocytes and stimulate the liver to produce acute phase proteins, which may have a role in the inactivation of bacteria (14). Of the cytokines, IL-1, TNF-α and interleukin-6 (IL-6) are the major mediators of acute phase protein synthesis (7).

Colostrum is the initial secretion in the lactation of the ewe. It contains immunoglobulins (Ig) which provide the lamb, which has little or no Ig present in its serum at birth, with passive immunity to certain infectious diseases (19). In ruminants, transfer of maternal immunity to the fetus is prevented by the placenta. Ruminants depend on Ig absorbed from colostrum for (passive) immunity prior to development of their own (active) immune systems (4). Neonatal morbidity and mortality decrease when adequate concentrations of Ig are received via colostrum (21). Neonates that fail to receive passive maternal immunity, normally obtained by ingestion of colostrum, are particularly susceptible to infection and sepsis (1).

*) This study was supported by Research Fund of Istanbul University. Project number: 1653/30042001.

Gram-negative septicemia is an important cause of mortality in sheep and failure of passive transfer of immunity by colostral ingestion has been positively correlated with sepsis. Serum TNF-α, IL-1β and IL-6 activities are used as a prognostic indicator for sheep with presumed sepsis (4, 6, 7).

The purpose of this study was to determine the relationship between TNF-α, IL-1β and IL-6 cytokines and experimental *M. haemolytica* infection in ewes that had been fed colostrum and dam milk or commercial cow milk after birth.

Material and methods

Animals. In this study, 20 one-year-old Chios ewes were used. Ten Chios new-born lambs were fed *ad libitum* colostrum and dam milk at their dam's side (control group). Other ten lambs were separated from their dams immediately. These lambs were not allowed to suckle normally and fed commercial cow milk with feeding bottle (experimental group). Each lamb was eartagged with individual identification numbers during the day following birth. When they were one-year-old, the study was conducted. Hay, concentrates and water were provided *ad libitum*. All ewes were determined to be clinically normal on the basis of results of physical examination at the beginning of the experimental period.

Preparation of *M. haemolytica*. *M. haemolytica*, serotype A1 (Cat. no: 103426T ATCC) was inoculated on brain-heart infusion agar plates and incubated at 37°C for 24 hours. Thereafter, the organisms were harvested with phosphate buffered saline (0.9% NaCl) (PBS) and washed with PBS three times. Bacteria were adjusted to 1×10^9 organisms/2 ml using sterile PBS. Inoculum was kept at 4°C.

Experimental design. Ewes (n = 20) were inoculated intratracheally with 1×10^9 log-phase *M. haemolytica* organisms suspended in 2 ml of PBS. Blood samples were collected from the jugular vein once before bacterial inoculation and 3 times at days 1, 10 and 22 after bacterial inoculation from all animals. Antibiotic treatment was applied at day 22. Blood samples were also collected at day 7 of treatment. Serum samples were stored in –70°C until analysed for TNF-α, IL-1β and IL-6 concentrations.

TNF-α, IL-1β and IL-6 assays. Serum TNF-α, IL-1α and IL-6 concentrations were estimated by a „sandwich-type" of enzyme immunoassay. A monoclonal anti human TNF-α, anti human IL-1β or anti human IL-6 antibodies are coated to polystyrene microtiter wells (PeliKine Compact Human ELISA, CLB, Amsterdam, The Netherlands). Coating antibody was brought to room temperature (18-25°C). Coating antibody was diluted 1 : 100 in coating buffer (0.1 M carbonate/bicarbonate) and added 100 µl to all wells. Plates were covered, incubated overnight at room temperature and washed five times with PBS. 200 µl blocking buffer was added to all wells. Plates were incubated for one hour at room temperature and washed five times with washing buffer (PBS with 0.005% TWEEN 20). 100 µl of standard and sample dilutions were added in triplicate to the appropriate wells by leaving the substrate blank wells empty. Biotinylated sheep TNF-α, IL-1β or IL-6 antibodies were diluted 1 : 100 in dilution buffer. Plates were washed five times with washing buffer. 100 µl of the diluted biotinylated antibody was added to all wells by leaving the substrate blank wells empty. Plates were covered and incubated one hour at room temperature. Streptavidin-horseradish peroxidase (streptavidin-HRP) conjugate was diluted 1 : 10 000 in dilution buffer. Plates were washed five times with washing buffer. 100 µl of the streptavidin-HRP conjugate was added to all wells by leaving the substrate blank wells empty. Plates were covered, incubated for 30 minutes at room temperature and washed five times with washing buffer. 100 µl substrate solution was added to all wells, including the substrate blank wells. Plates were incubated for 30 minutes at room temperature in the dark. 100 µl stop solution (1.8 M H_2SO_4) was added to all wells and plates were read at 450 nm in microtiter plate reader (EL × 800 Microplate reader, Bio-Tek Instruments, Inc.). From the absorbance of samples and those of a standard curve, the concentrations of serum TNF-α, IL-1β and IL-6 were determined by inter polation with the standard curve.

Statistical analysis. Mean serum TNF-α, IL-1β and IL-6 concentrations for control and experimental groups in each blood sampling day were compared by use of a two-tailed Student's t-test. Data were analysed by use of factorial and repeated measure ANOVA between blood sampling days within each group using a computer program (SPSS, version 11.5). Results are presented as mean ± S.E.

Results and discussion

Serum TNF-α concentrations of control and experimental groups were presented in fig. 1. A significant difference could not be determined between blood sampling days in both groups in the study. A rise was observed at day 1 postinfection. In study of Horadagoda and Eckersall (9), serum TNF-α concentration rose to a peak two hours after inoculation, ranging from 18 to 37 ng/ml in all *M. haemolytica*-infected calves. The levels fell rapidly four to eight hours after inoculation. However, Pace et al. (18), using a cytotoxic method, demonstrated an increase in circulating TNF-α after the intra-tracheal inoculation of *M. haemolytica*, which began within two hours of challenge and lasted for 48 to 72 hours in calves. Serum TNF-α levels increased to maximum 6 ng/ml and peaked at 8 h post inocula-

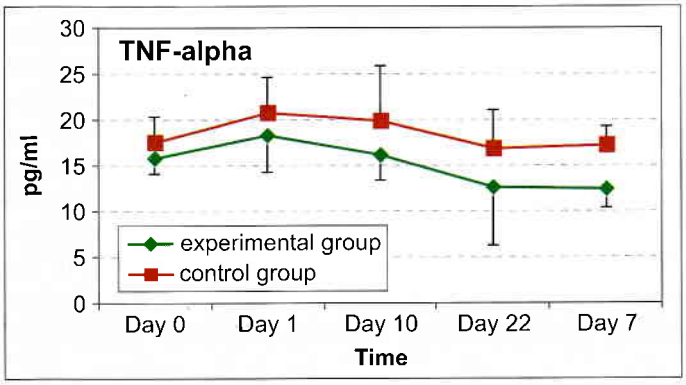

Fig. 1. The changes in serum TNF-α levels through the study (mean ± SE)

tion. In contrast, Espinasse et al. (3), using a radioimmunoassay, were unable to detect increased serum TNF-α concentrations in calves exposed to repeated nasal and intra-tracheal inoculations of *M. haemolytica*.

That a significant increase in serum TNF-α levels was not detected in the study is thought to be because adequate TNF-α response could not be obtained, caused by relatively rare specimen collecting schema when compared with other studies. However, another probability was a possible insufficiency in the microbial dose given. For example, 1×10^{10} *M. haemolytica* A1 were used in this study, although in study of Horadagoda and Eckersall (9) obtaining increase in serum TNF-α levels 4×10^{10} microorganisms were used.

In a study, lipopolysaccharides from *Escherichia coli* were infused to neonatal foals; postinfection serum TNF-α concentrations peaked later and were lower in colostrum-deprived foals than colostrum-fed group, but a significant difference was not found in serum TNF-α concentrations between colostrum-fed and colostrum-deprived foals (1).

As similar to findings of Allen et al. (1), in this study, serum TNF-α levels were insignificantly higher in group ingesting colostrum and dam milk than other group. The reason of this must be immunostimulatory effect of colostrum ingestion that serves to increase resistance to infection in neonates (20). No significant difference between two groups may be because the colostrum and dam milk-deprived group relatively lost the difference on resistance to infections within one year postpartum when compared with the other group.

Serum IL-6 concentrations of control and experimental groups were presented in fig. 2. In this study, serum IL-6 levels slightly rised one day after the start of the infection and gradually diminished in both groups. High concentrations of IL-6 have been measured in serum and body fluids in human beings after the administration of endotoxin, TNF-α, and IL-1, and during acute bacterial infection and sepsis (8). In cases of feline infectious peritonitis significant IL-6 activity was found in ascitic fluid and in serum (5). After endotoxin administration to pigs, plasma concentrations of IL-6 increased (12). A significant increase in serum IL-6 activity was determined between 1 to 8 hours after endotoxin infusion to horses (2). In another study, serum IL-6 activity significantly increased from 1 through 5 hours after start of infusion and peaked at 3-4 hours in horses given endotoxin (15). The significant increase observed in other studies could not be determined in this study. The reason of this is possibly that the blood sampling was 24 hour after inoculation. When examined other studies, it was observed that peak levels of serum IL-6 coincided with 4-5 hours after infusion. In a study investigating IL-6 concentrations after bacterial lipopolysaccharide infusion in adult horses, IL-6 activity was found in pre-infusion plasma samples (11). However, in another study, IL-6 activity was not determined preinfusion serum samples (15). In study of Robinson et al. (20) IL-6 in low concentrations was detected in serum samples from 2 of 8 colostrum-deprived foals before infusion of *Escherichia coli* lipopolysaccharide. In the present study, a baseline serum IL-6 activity was detected before bacterial inoculation. These results may be attributed to differences in assay sensitivity between different laboratories (20). Moreover, in study of Robinson et al. (20), serum IL-6 concentration was significantly higher than baseline concentration by 30 minutes in colostrum-fed foals and by 45 minutes in colostrum-deprived foals given *Escherichia coli* lipopolysaccharide. Peak concentrations were recorded 90 minutes after infusion of lipopolysaccharide in colostrum-fed foals and 120 minutes after infusion in colostrum-deprived foals. Colostrum-deprived foals had lower serum IL-6 concentrations than colostrum-fed foals. Also, in the present study, serum IL-6 levels were relatively found lower in colostrum and dam milk-deprived ewes than other group.

Serum IL-1β concentrations of control and experimental groups were presented in fig. 3. IL-1 elicits immediate local inflammatory reactions that are characterized by binding of blood neutrophils to endothelial cells, followed by infiltration and oedema (14). It invokes B-cell activation, antibody synthesis, fever,

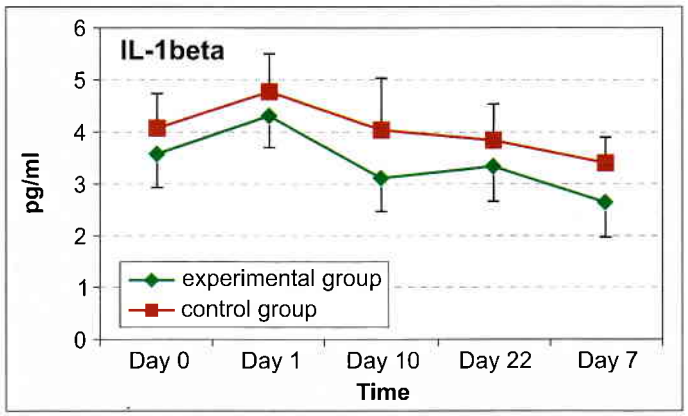

Fig. 2. The changes in serum IL-1β levels through the study (mean ± SE)

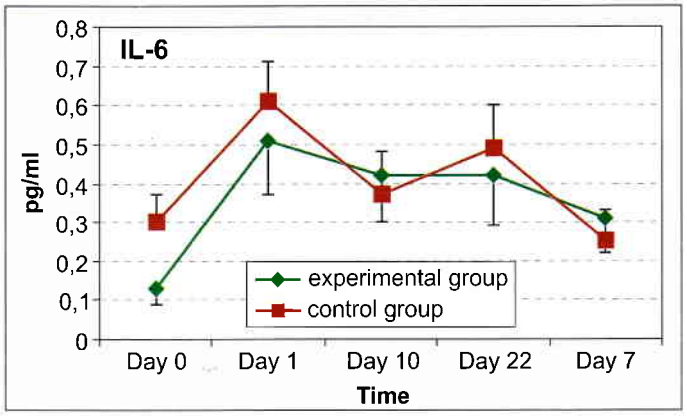

Fig. 3. The changes in serum IL-6 levels through the study (mean ± SE)

secretion of inflammatory proteins and release of prostaglandins (6, 14). In a study, serum IL-1 activities were not detected in calves and lambs before infection (17). Moreover, Nakamura (16), in his study, did not detect serum IL-1 level in control rabbits. However, in the present study, a baseline IL-1 level was found in ewe sera before infection. The reason of this difference may be different experimental circumstances as reported before. In another study, IL-1β significantly increased in lung lesions of calves infected by *M. haemolytica* (13). Peak concentrations occurred within 16 hours post infection. Alveolar and interstitial macrophages produced IL-1b in first 4 hours, and by 8 hours post infection, neutrophils were the dominant source of IL-1β. In another study, increased levels of bioactive IL-1 were found in lavage fluids from *M. haemolytica* – infected calves (22). Nakamura (17), in his study, infected calves and lambs with *Strongyloides papillosus* and found detectable IL-1 activities in five animals, including one calf that survived. Most of animals that died had no IL-1 production. Moreover Nakamura (16), in his another study, detected serum IL-1 levels between 1 and 10 weeks post infection in 4 rabbits infected with *Trypanosoma brucei brucei*, with maximum values ranging from 17.0 to 245.2 U/ml. In the present study, as in TNF-α and IL-6 levels, serum IL-1 levels slightly rose at day 1 post infection and then lowered. This insignificant rising may be attributed to paucity of sampling frequency or insufficiency of bacterial dose as reported before.

In conclusion, inflammatory cytokines, i.e. TNF-α, IL-β, and IL-6, may play a role in the pathogenesis of lung injury in pneumonic pasteurellosis of lambs. Colostral factors, acting either directly or indirectly, enhance immune response mechanisms of colostrum-fed lambs thereby contributing to the defense against infections after parturition. The reduced responses observed in the colostrum and dam milk-deprived ewes may be a result of absence of this effect. The results may indicate that the inflammatory response of ewes fed colostrum and dam milk after birth is relatively more potent than of ewes fed artificially. Moreover, non significant differences between two groups indicate that a resistance against infections developed in colostrum-deprived animals within one year time.

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