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Effects of EDTA-Induced Hypocalcaemia and Stress on Plasma TNF-α, IL-1-ra, G-CSF, GM-CSF and S-100 in Dairy Cows

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ABSTRACT

The pathophysiology of postparturient paresis is still not completely understood. Knowledge recently acquired in immunology, endocrinology and cell physiology has still to be integrated in order to elucidate the aetiopathogenesis of the disease. For that purpose, the effect of the EDTA infusion model on the plasma concentrations of selected cytokines and growth factors, and of a calcium binding protein was examined in dairy cows. Six 6- to 11-year-old Brown Swiss cows in mid lactation were infused with a 5% solution of Na_2EDTA in one jugular vein over a period of 5 h. Blood samples were collected from the contralateral side daily two days before, and then hourly for five hours during the infusion, hourly for five hours after the end of the infusion, and once daily for 10 days thereafter. The plasma concentrations of cortisol, tumour necrosis factor-α, interleukin-1 receptor antagonist, granulocyte colony-stimulating factor, granulocyte and macrophage colony-stimulating factor, and the calcium binding protein S-100 were determined. Before the EDTA infusion, during the infusion and for two days thereafter, the mean plasma concentrations of cortisol were significantly higher than those from days 4 to 10 after the infusion. The plasma concentrations of tumour necrosis factor-α and interleukin-1 receptor antagonist followed a similar profile. At the end of EDTA infusion, low concentrations of granulocyte colony-stimulating factor were detected in one cow only. On days 3 and 4, the mean plasma concentrations of granulocyte colony-stimulating factor were significantly higher than the pre-infusion values, but this was followed by a significant decrease on post-infusion day 5. From day 4 to 7, the plasma concentrations of S-100 were significantly lower than the pre-infusion values. The importance of these findings in the pathophysiology of postparturient paresis remains to be established.

Keywords: calcium binding protein; colony-stimulating factor, cortisol, cow, cytokines, ethylenediaminetetraacetate, interleukin receptor antagonist, stress, tumour necrosis factor

Abbreviations: S-100, calcium binding protein S-100; EDTA, ethylenediaminetetracetate; GM-CSF, granulocyte and macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; IL-1-ra, interleukin-1 receptor antagonist; TNF-α, tumour necrosis factor-α

INTRODUCTION

Postparturient hypocalcaemia of dairy cows is a commonly encountered disease with a complex pathogenesis (Goff *et al.*, 1991; Horst *et al.*, 1997). Many aspects of its pathophysiology have been investigated with the EDTA infusion model (van de Braak *et al.*, 1984; Desmecht *et al.*, 1995, 1996; Riond *et al.*, 1997). However, knowledge recently acquired in immunology, endocrinology and cell physiology has still to be integrated to fully elucidate the aetiopathogenesis of the disease. Furthermore, the immune system is suppressed during the periparturient period (Kehrli and Goff, 1989). Many cytokines and growth factors are important modulators of the immune responses and are also regulators of a variety of organ functions that are pivotal for calcium homeostasis, for example, bone remodelling (Mundy, 1993). A possible involvement of cytokines, growth factors and calcium binding proteins in the pathophysiology of postparturient hypocalcaemia has not been examined so far. Extensive reviews exist of the biological regulatory activities of tumour necrosis factor-α (Aggarwal and Natarajan, 1996; Bazzoni and Beutler, 1996; Hill and Lunec, 1996), interleukin-1 receptor antagonist (Nikolic-Paterson *et al.*, 1996; Mantovani *et al.*, 1996), granulocyte colony-stimulating factor (Hansson and Söderström, 1993; Metcalf, 1997), granulocyte and macrophage colony-stimulating factor, and the calcium binding protein S-100 (Zimmer *et al.*, 1995; Schäfer and Heizmann, 1996). The purpose of this study was to investigate the effects of EDTA-induced hypocalcaemia on the serum concentrations of TNF-α, IL-1-ra, G-CSF, GM-CSF and S-100.

MATERIALS AND METHODS

Animals and treatments

Six Brown Swiss cows, aged 6 to 11 years, in mid-lactation, weighing 610 ± 22 (SD) kg and with an average daily milk production of 22.5 ± 2.0 L, were used. The animals were housed in a closed barn and were fed 30 kg of a mixture of 2/3 grass silage, 1/6 corn silage, 1/6 beet chips, and 3 kg of concentrate in two daily portions at 06:00 and 17:00. The daily ration provided 100.1 MJ net energy for lactation, 1359 g absorbable protein in the intestine, 114 g calcium, 55 g phosphorus and 28 g magnesium. The cows were milked between 06:00 and 06:30 and between 17:00 and 17:30. After 2 days of acclimatization to a new place within the barn, a 5% solution of disodium ethylenediaminetetracetate (Na_2EDTA) was infused via a catheter in the right jugular vein at 0.90 mmol/min for 5 h. Blood was collected from the contralateral side via a catheter into blood tubes that contained potassium oxalate as an anticoagulant and sodium fluoride as a glycolytic inhibitor, daily 2 days before, shortly after and then hourly for 5 h during the infusion and hourly for 5 h after the end of the infusion. The catheters were implanted 1 day before the infusion. Further blood samples were collected twice daily on days 1 and 2 and then daily for 8 days thereafter. The blood was subsequently centrifuged at 1580*g* for 10 min at 4°C and the plasma was aspirated. The samples were then stored at 4°C and analysed within 1 week.

Biochemical analyses

The concentration of cortisol in plasma was determined by a radioimmunoassay (RIA) as previously described (Bahr *et al.*, 1998). The sample (100 µl) was extracted in duplicate with ethanol (2.0 ml) for 30 min. The tubes with the precipitated proteins in them were centrifuged at 4°C for 15 min and the ethanolic supernatants were decanted into incubation tubes. After evaporation of the organic solvent with nitrogen, the residues were reconstituted in 300 µl of assay buffer. Immunoreactive cortisol titres were determined by a cortisol RIA that included an iodinated tracer and a double-antibody separation of bound and unbound ligand, and phosphate-buffered saline (0.01 mol/L, pH 7.0) with 0.1% gelatin as the assay buffer.

The concentrations of G-CSF, GM-CSF and IL-1-ra were determined using commercially available enzyme immunoassays (Quantikine Kit from R&D Systems, Minneapolis, MN, USA). The quantity of TNF-α was measured by an enzyme-linked immunosorbent assay (Bioassay TNF-α US ELISA Kit ultrasensitive, NBS Biologicals Ltd, Cambs, UK) and S-100 by a luminescence-immunoassay (LIA-mat Sangtec-100, BYK-Sangtec Diagnostica GmbH, Germany).

Statistical analyses

The changes with time in the concentrations of cortisol, TNF-α, IL-1-ra, G-CSF, GM-CSF and S-100 were examined by repeated measurements analysis of variance using version 6.11 of the mainframe computer-implemented SAS procedure GLM (SAS, 1989). For each parameter, the values of the concentrations during and after the infusion were compared with either the first or second pre-infusion value by use of the option *contrast* and adjacent levels of the parameters were compared by use of the option *profile*. The level of significance was fixed at $p < 0.05$.

RESULTS

The pathophysiological effects and the changes in the serum concentrations of total and ionized calcium and inorganic phosphate induced by the EDTA infusion have been reported elsewhere (Riond *et al.*, 1997). The data (mean ± SD) for cortisol, cytokines, growth factors and S-100 are presented in Figure 1A–F. During the days before the EDTA infusion, during the infusion and for 2 days thereafter, the mean plasma concentrations of cortisol were significantly higher than those from day 4 to day 10 after the infusion (Figure 1A). The SAS option *contrast* indicated a significant decrease in cortisol concentrations by day 3. The plasma concentrations of TNF-α and IL-1-ra followed a similar profile with significantly lower concentrations on post-infusion day 5 (Figure 1B–C). The SAS option *profile* revealed decreasing concentrations on day 2 for TNF-α and in the first part of day 1 for IL-1. During the last 2 h of the EDTA infusion, low concentrations of G-CSF were detected in one cow (Figure 1D). On days 3 and 4, the mean serum concentrations of G-CSF were significantly

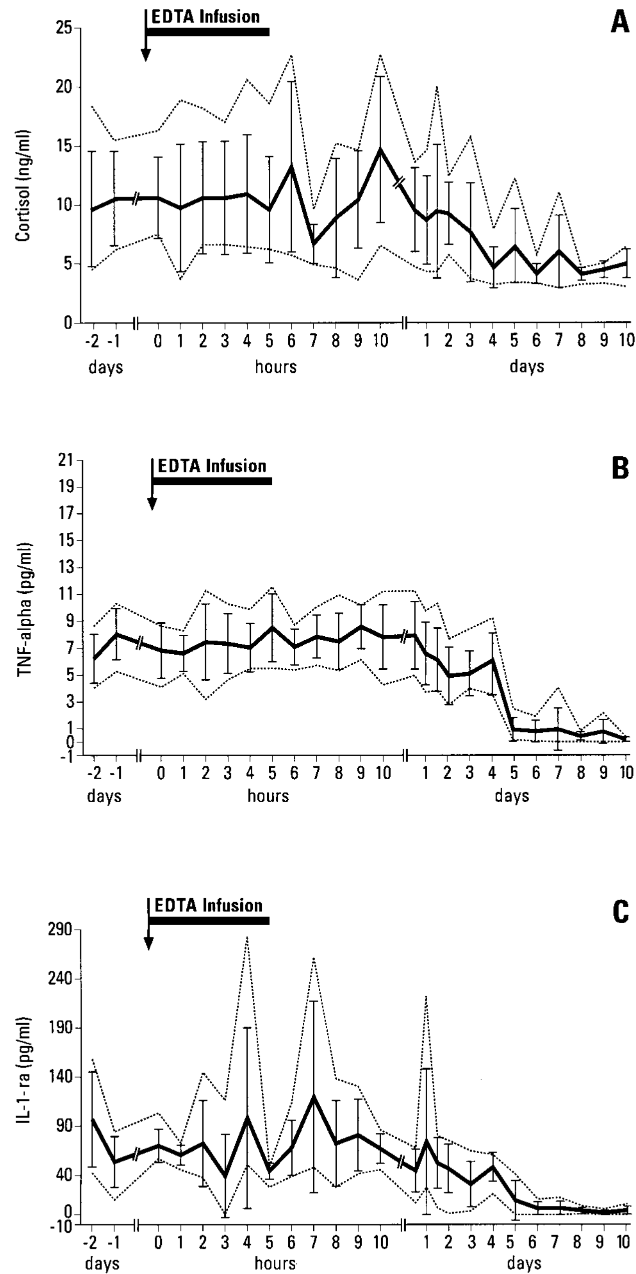

Figure 1. The effects of EDTA infusion and stress on the plasma concentrations (means ± SD with minima and maxima shown as dotted lines) of (A) cortisol, (B) TNF-α, (C) IL-1-ra, (D) G-CSF, (E) GM-CSF and (F) S-100 in six dairy cows

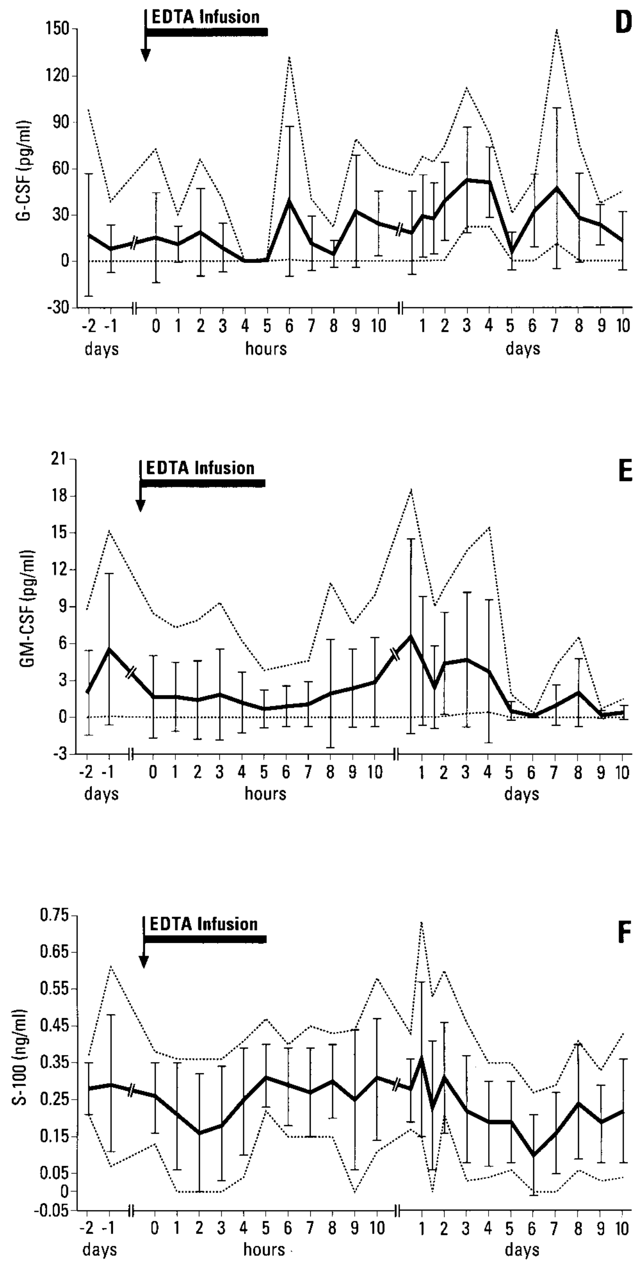

Figure 1. See legend on opposite page

higher than the pre-infusion values, but this was followed by a significant decrease on day 5. No significant changes with time were observed in the concentrations of GM-CSF (Figure 1E). From days 4 to 7, the serum concentrations of S-100 were significantly lower than the pre-infusion values (Figure 1F).

DISCUSSION

As the plasma concentrations of TNF-α and IL-1-ra, which were not directly related to the EDTA infusion, were unexpectedly elevated during the first part of the experiment, the cortisol concentrations were measured retrospectively. Psychological stress is the most likely cause of the elevated plasma concentrations of TNF-α, IL-1-ra and cortisol following activation of the sympathetic/adrenomedullary system with subsequent systemic release of the inflammatory cytokines IL-6, IL-1 and TNF-α (Bellinger *et al.*, 1992; Stratakis and Chrousos, 1995). The inflammatory cytokines are known to activate the hypothalamic–pituitary–adrenal axis, with subsequent elevation of the concentrations of ACTH and cortisol (Besedovsky *et al.*, 1986; Sapolsky *et al.*, 1987; Perlstein *et al.*, 1993; Woloski *et al.*, 1985; Mastorakos *et al.*, 1993). Glucocorticoids in turn suppress the stimulatory effects of cytokines on the hypothalamic–pituitary–adrenal axis (Lázár *et al.*, 1992; Besedovsky *et al.*, 1986; Beutler *et al.*, 1986; Dubois *et al.*, 1989). In the present study, the concentrations of IL-1 and IL-6 were not determined. The elevated plasma concentrations of IL-1-ra before, during and for 4 days after the infusion may be explained by the fact that increased production of IL-1-ra is usually associated with an increased production of IL-1 (Mantavoni *et al.*, 1996; Nikolic-Paterson *et al.*, 1996). The source of the stress for these cows was probably their accommodation in a new place in the barn and/or new people. In contrast to the present study, cortisol concentrations increased significantly in assocation with EDTA infusion when the EDTA was infused at twice the rate (Desmecht *et al.*, 1996) and cortisol concentrations were also significantly increased in postparturient cows with severe hypocalcaemia (Horst and Jorgensen, 1982). To properly evaluate the degree of the stress, it would be better to make serial determinations of cortisol plasma concentrations in the course of the day because episodic secretion, inducing large fluctuations of cortisol concentrations, occurs in cattle (Thun *et al.*, 1981; Thun, 1986, 1987). Furthermore, a pronounced circadian rhythm has been observed for plasma cortisol. In the present study, TNF-α and IL-1-ra concentrations decreased significantly before the cortisol concentrations did so. This observation confirms the controlling role of these two cytokines on the adrenomedullary axis.

The decreased G-CSF serum concentrations following the EDTA infusion may be the consequence of hypocalcaemia or hypophosphataemia or of the chelation of other ions, such as zinc. The relatively small volume of the carrier saline solution in comparison to the volume of the extracellular space is unlikely to have induced changes in the extracellular fluid that would explain such a change in the concentration of G-CSF. At the end of the infusion, the production of G-CSF by monocytes and endothelial cells was possibly decreased or their elimination may have been enhanced. The importance of this finding in the pathophysiology of postparturient paresis of dairy cows remains to be established.

The period over which S-100 concentrations were significantly decreased corresponds to the beginning of the period with low concentrations in the serum of cortisol, TNF-α and IL-1-ra. This observation suggests a transient effect from low concentrations of cortisol, TNF-α and/or IL-1-ra which lasts only until a readjustment has occurred, following feedback mechanisms.

CONCLUSION

Kits developed for the determination of human TNF-α, IL-1-ra, G-CSF, GM-CSF and S-100 in plasma may be used for cattle. The observations on the changes in the plasma concentrations of cortisol, TNF-α, IL-1-ra, G-CSF, GM-CSF and S-100 in relation to the infusion of EDTA may be useful for the design of future trials, which are still needed for a better understanding of the pathophysiology of postparturient paresis in dairy cows. This study strongly suggests an involvement of TNF-α and IL-1-ra in the stress response of cows. These two cytokines could be used as markers for stress in cattle. Also, the importance of stress as a confounding factor should not be neglected in this type of investigation. Finally, the role of G-CSF in postparturient paresis remains to be established.

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