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Sensitization of the bovine mammary gland to *Escherichia coli* endotoxin

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**Summary** — The effect of repeated infusions of *Escherichia coli* endotoxin on the acute phase response in the bovine mammary gland was assessed through the concentrations of tumor necrosis factor-α (TNF-α) in milk. Four clinically normal lactating cows received two intramammary infusions of *E. coli* endotoxin (33 μg) 24 h apart in the same mammary quarter. Along with the second infusion, the cows received one dose of endotoxin in the contralateral quarter. Milk was collected at varying intervals before and after infusion and TNF-α concentrations were determined by ELISA. Following the first infusion at 0 h, the mean concentrations of TNF-α augmented from undetectable concentrations to a maximum of 0.4 ng/mL at 4 h and declined to below 0.04 ng/mL at 24 h, the time of the second infusion. In the quarters challenged twice, the increase in TNF-α concentrations was abrupt, culminating at 11.7 ng/mL 6 h later (at 30 h). The increases in TNF-α concentrations were similar in the contralateral quarters infused once. TNF-α concentrations in the control, uninfused quarters of infused cows remained undetectable (< 0.04 ng/mL). Despite the low TNF-α response following the first infusion, mean somatic cell counts increased markedly, being only slightly lower than after the second infusion (107/mL and 5 × 107/mL at 8 h and 32 h, respectively) in the quarters challenged twice. After the first infusion, none of the cows developed fever, but following the second infusion, rectal temperature increased markedly, culminating 6 h after the second infusion. These results show that an infusion in one quarter of an amount of endotoxin sufficient to induce a pronounced cell recruitment but insufficient to induce a marked TNF-α secretion following the first infusion sensitized not only that quarter but also the contralateral one to a second infusion with the endotoxin. It is thus possible that sensitization of the whole udder follows a first contact with a moderate dose of endotoxin in one quarter.

endotoxin / tumor necrosis factor-α / mastitis / bovine / inflammation

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Résumé — Sensibilisation de la glande mammaire de vache à l’endotoxine d’Escherichia coli.

Les variations de concentrations de TNF-α dans le lait ont été utilisées pour évaluer l’effet d’injections répétées d’endotoxine de Escherichia coli sur la réaction inflammatoire aigüe de la glande mammaire de la vache. Quatre vaches Holstein cliniquement normales ont reçu deux injections intramammaries d’endotoxine (33 μg) à 24 heures d’intervalle dans le même quartier. Au moment de la deuxième injection, une dose d’endotoxine a également été administrée dans le quartier controlatéral. Du lait a été prélevé à intervalles variables avant et après l’injection, et les concentrations de TNF-α ont été mesurées par ELISA. Après la première injection, les concentrations moyennes sont passées de valeurs indétectables à un maximum de 0,4 ng/mL à 4 heures post-injection, puis sont redescendues en-dessous de 0,04 ng/mL à 24 heures, le moment de la deuxième injection. Dans les quartiers éprouvés deux fois, l’élévation de concentration de TNF-α a été brutale, culminant à 11,7 ng/mL 6 heures après la deuxième injection (à 30 heures). Les variations de concentrations de TNF-α dans les quartiers controlatéraux infusés une seule fois ont été comparables. Les concentrations de TNF-α dans les quartiers non éprouvés de ces animaux sont restées indétectables (< 0,04 ng/mL). Bien que l’accroissement des concentrations de TNF-α après la première injection ait été modeste, la numération cellulaire du lait a fortement augmenté, n’étant seulement qu’un peu inférieure aux valeurs enregistrées après la deuxième injection (107/ml et 5 x 107/ml à 8 heures et 32 heures, respectivement), dans les quartiers éprouvés deux fois. La première infusion d’endotoxine n’a pas induit de fièvre, mais la seconde infusion a provoqué une forte hyperthermie qui a atteint un maximum 6 heures plus tard. Ces résultats indiquent que l’injection dans un quartier d’une quantité d’endotoxine suffisante pour induire un afflux cellulaire important mais insuffisante pour provoquer une sécrétion forte de TNF-α, a sensibilisé non seulement le quartier injecté mais aussi le quartier controlatéral à une deuxième injection d’endotoxine. Il est donc possible que la sensibilisation de la mamelle entière résulte d’un premier contact avec une dose modérée d’endotoxine dans un de ses quartiers.

endotoxine / facteur nécrosant des tumeurs-α / mammite / vache / inflammation

INTRODUCTION

Mastitis caused by coliform organisms remains common in dairy cows. This is partly because the prophylactic measures (teat dipping and antibiotic therapy at drying off), which proved of value to combat contagious mastitis, are not very efficient against environmental mastitis (Eberhart, 1977). Usually the quarter suffering from coliform mastitis undergoes a clinical episode followed by a spontaneous bacteriological cure, and a marked temporary loss of milk production. Sometimes the infection is peracute, and life-threatening. In every case the economic losses are sizeable.

It is thought that after the penetration of Escherichia coli through the teat canal into the lumen of the mammary gland, the multiplication of bacteria sets off a potent inflammatory response, which usually rids the gland of the bacteria (Hill et al, 1978). The bacterial component which is most likely responsible for the characteristic severity of the inflammation accompanying coliform mastitis is endotoxin, more specifically its lipid A moiety, the toxic part of the molecule (Morrison, 1983). Acute mastitis with systemic manifestations has been produced by the intramammary infusion of endotoxin from Klebsiella and E coli (Carroll et al, 1964; Paape et al, 1974). It is for this reason that endotoxin has been widely used over the years as an experimental challenge model to study the inflammatory response of the cow’s udder, and in particular to determine which inflammatory mediators contribute to the reaction (Anderson et al, 1986; Shuster et al, 1993).

Particular attention has been paid to tumor necrosis factor α (TNF-α), because this cytokine, along with interleukin 1 (IL-
appears to be especially important in triggering the acute phase response (Bau-
mann and Gauldie, 1994). TNF-α has been found in milk during endotoxin-induced or E coli mastitis (Sordillo and Peel, 1992; Shuster and Kehrli, 1995). Nevertheless, elevated TNF-α concentrations in milk appeared to be only associated with the most severe manifestations of coliform mastitis (Sordillo and Peel, 1992) or induced by relatively high doses of endotoxin (Shuster et al, 1993; Shuster and Kehrli, 1995), suggesting that the magnitude of TNF-α secre-
tion in milk is commensurate with the intensity of the inflammatory response.

Although previously reported studies suggest that the concentrations of TNF-α in milk depend on the amount of endotoxin infused intramammarily, there is no information on the effect that repeated injections of endotoxin could have. In the course of an intramammary infection by E coli, the shedding of endotoxin in milk most likely differs from the kinetics achieved by the infusion of a single large dose. On the contrary, the initiation of the inflammatory response could be triggered by either several small doses of endotoxin or even by a continuous release of minute amounts of endotoxin in milk. In this study we reported the variations in the TNF-α concentrations in milk following two consecutive intramammary infusions of a moderate dose of endotoxin, with a view to improving our understanding of the inflammation process accompanying E coli mastitis.

MATERIALS AND METHODS

Experimental design

Four clinically healthy lactating cows were selected on the basis of the results of bacteriologic testing of foremilk samples. The samples were collected aseptically and examined according to the bacteriologic procedures outlined in the National Mastitis Council’s handbook (Har-
mon et al, 1990). At the beginning of the experi-
ment, all the quarters were free from infection, except for one control quarter, infected by a coag-
ulase-negative Staphylococcus. No new infec-
tions appeared during the experimental period.

A suspension of 2 μg/mL of E.coli O128:B12 lipopolysaccharide (Difco Laboratories, Detroit, Michigan, USA) in 0.85% saline solution was sterile-filtered (0.2 μm). After filtration, 1.32 μg/mL of endotoxin remained in the filtered suspension, as calculated with a Limulus ame-
boocyte lysate assay (QCL-1000; Bio-Whittaker, Walkersville, Md). A dose of 33 μg endotoxin in 25 mL of saline was infused into one fore quarter of each cow after the morning milking, using a 14-gauge cannula and syringe. Quarter milk samples were collected by hand just before the infusion and at post-infusion (PI) hours: 1, 2, 4, 6 and 8. After the next morning milking (PI 24), milk was collected and a second infusion of endotoxin (same dosage as the first one) was administered into the two fore quarters of each animal, so that one quarter received two infu-
sions 24 h apart, and one quarter received only one (the dose at PI 24). Then milk samples were collected at PI at hours 25, 26, 28, 30, 32 and 48 (with reference to the first infusion).

A portion of the samples was used to deter-
mine the total milk somatic cell counts (SCC) using an automated cell counter (Fossomatic model 90, Foss Food Technology, Hillerod, Den-
mark), according to the procedure of Miller et al (1986) for fresh milk samples. Another por-
tion was centrifuged at 44 000 × g for 10 min at 4 °C to remove the cream and the cell pellet. Whey was prepared by centrifuging the skimmed milk at 44 000 × g for 55 min at 4 °C. The opales-
cent upper layer was aspirated and the clear supernatant was stored in portions at -20 °C.

At each sampling time, the rectal tempera-
ture was taken and a clinical examination was performed (appearance of gland and milk).

Enzyme-linked immunosorbent assay (ELISA) for TNF-α

Flat-bottom microtiter plates (Immulon 2; Dynat-
ech) were coated with 100 μL of goat anti-mouse immunoglobulin (Jackson Immunoresearch Labora-
atories, West Grove, Pa, USA) at 2 μg/mL in carbonate-bicarbonate buffer, pH 9.6 for 90 min at 37 °C. After two washes with 0.01 M phos-
phate-buffered 0.85% saline, pH 7.4 supple-
mented with 0.01% Tween 20 (PBST), the
remaining binding sites were blocked with 200 μL of 0.5% gelatin for 30 min at 37 °C. The plates were washed twice and 100 μL of monoclonal antibody anti-bovine TNF-α (2C4 ID3; Malstrom CE, Paape MJ and Elsasser TH, unpublished results) diluted 1/10,000 in PBST plus 0.1% gelatin (PBSTG) was distributed in the wells and incubated for 1 h at 37 °C. After three washes, 100 μL of appropriate dilutions (usually 1/5 or 1/10) of samples to be tested and a series of twofold dilutions of recombinant bovine TNF-α (from 10 ng/mL to 0.39 ng/mL; provided by Dr Ramp, CIBA-GEIGY, Basel, Switzerland) were added and incubated for 1 h at 37 °C. The plates were washed three times and rabbit serum anti-TNF-α (Kenison et al., 1990) at 1/5000 in PBSTG was incubated for 30 min at 37 °C. After three washes, the plates were incubated for 30 min with goat anti-rabbit Ig conjugated with peroxidase at 1/10,000. Finally, the plates were washed, the enzyme substrate, 52 mM 2,2’-azino-di-(3-ethylbenzthiazoline-sulfonate; Boehringer GmbH, Mannheim, Germany) in 0.1 M citrate buffer pH 4.2 with 0.075% hydrogen peroxide, was added just before use. The absorbance was read at 415 nm with an ELISA plate reader after about 30 min at room temperature. Wells that received PBS instead of monoclonal antibody served as blanks. TNF-α concentrations were calculated by referring to a standard curve.

RESULTS

Clinical signs of mastitis, principally udder swelling, developed approximately 4 h after the first endotoxin infusion. Udder swelling was more pronounced and began earlier (within 2 h) after the second infusion of endotoxin. Occasional clots were seen in milk during the first day PI, but on the second day large clots appeared within 2 to 4 h in the milk from the quarters infused twice, and the milk became yellowish. In two quarters infused once (at PI 24) milk had a yellowish appearance by PI 28, and at PI 32, all infused quarters delivered a yellowish secretion.

After the first infusion, none of the cows developed fever, but following the second infusion, a systemic reaction accompanied the mammary inflammation, as demonstrated by increases in the rectal temperature which culminated 6 h after the second infusion (fig 1). The temperature returned to normal by PI 48 (fig 1).

The somatic cell counts began to increase in the infused quarters by PI 4, and reached a plateau at around 10⁷ cells/mL by PI 8 (fig 2). The cell counts remained high until the second infusion, which was accompanied by a further moderate rise to 5 x 10⁷ cells/mL at PI 32 (fig 2). Cell counts remained low in the uninfused quarters. In the quarters receiving only one infusion at PI 24, the cell recruitment began 4 h after infusion and continued to increase up to PI 48. At the end of the follow-up period, the quarters having received one infusion had cell counts comparable to those of the quarters infused twice (fig 2).

The concentrations of TNF-α in the milk increased slightly after the first infusion, rising from less than 0.04 ng/mL (lower limit of detection of the assay) to about 0.4 ng/mL at PI 4 (fig 3). In contrast, after the second infusion of endotoxin, the TNF-α concentrations began to increase in the milk of the challenged quarters as early as 1 h after the second infusion, and reached average values of 11.7 ng/mL at PI 30 (range: 1.5 to 25 ng/mL) for the quarters infused twice (fig 3). The kinetics of TNF-α increases were quite similar after the second infusion in quarters infused once, and the peak values were comparable. The concentrations returned to normal in the quarters infused twice by PI 48, or were still on the decline in the quarters infused once. The concentrations of TNF-α remained below the lower limit of detection of the assay in the uninfused quarters.

DISCUSSION

Following the first infusion of endotoxin, a local inflammatory reaction developed, with
Fig 1. Rectal temperatures of the four experimental cows, relative to the injection of 33 μg of endotoxin at post-infusion (Pl) hour 0 and Pl 24. Individual values are given. Arrows indicate time of infusion with endotoxin.

Fig 2. Somatic cell counts in milk obtained from quarters infused either twice with endotoxin (○), once 24 h after the first infusion (Pl 24) (□), or non-infused (●). Each of the four cows received one dose (33 μg) of endotoxin into one fore quarter at Pl 0, and one dose into each fore quarter at Pl 24. One uninfused rear quarter served as control. Bars indicate standard error of the mean. Arrows indicate time of infusion with endotoxin.
a massive influx of leukocytes, but no apparent systemic reaction (fever) occurred and the level of TNF-α in milk whey was barely detectable. The lack of a temperature increase may seem surprising, considering that in previously published work, comparable doses of endotoxin induced pyrexia. A possible explanation for the lack of hyperthermia is that endotoxin preparations differ in potency. Using the same batch as used in the present work in a cooperative study including the USDA and FDA on 35 clinically normal lactating cows, elevated body temperatures were never observed after intramammary injection of 10 μg E. coli endotoxin (S. Sechen, N. Alderson, and MJ Paape, 1995, unpublished data). Whatever the reason for the lack of fever, it is remarkable that the first infusion of endotoxin provoked a striking cell count response.

It has previously been noted that a relatively low dose of endotoxin (10 μg) can induce a local inflammation without involving detectable increases in TNF-α concentrations (Shuster et al., 1993). Higher doses, however, do induce TNF-α secretion in milk (Lahti et al., 1994; Shuster and Kehrli, 1995), and the highest TNF-α concentrations occur in the milk of the most severe cases of E. coli mastitis (Sordillo and Peel, 1992). These observations suggest that a strong inflammatory reaction is necessary to attain detectable amounts of TNF-α in milk, and that the dose of endotoxin preparation used in the present study was below the critical amount.

A second infusion of the same dose of endotoxin 24 h later, when the initial local inflammation had not yet abated, reinforced

Fig 3. Mean concentrations of TNF-α in whey prepared from uninfused control quarters (●), quarters infused twice with endotoxin (○), or only once at PI 24 (□). Bars indicate standard error of the mean, arrows indicate time of infusion with endotoxin.
the local manifestations of inflammation (inducing major alteration to the milk), and remarkably induced a strong secretion of TNF-α in milk of all cows. The TNF-α secretion was not limited to the previously challenged quarters, but also occurred in the quarters receiving only one infusion on the second day. Nevertheless, local signs of inflammation and the presence of TNF-α in milk did not generalize to the whole gland, since milk from the control uninfused quarters remained normal. This result showed that after an initial contact with a moderate dose of endotoxin, the whole mammary gland became more responsive to a second stimulation, so that a dose of endotoxin which was insufficient to provoke a sizeable TNF-α secretion in milk induced a strong local response accompanied by fever. Contrary to the first infusion, the second infusion of endotoxin provoked fever, as was shown by the increase in rectal temperature. This could result from the doubling of the total amount of endotoxin received by the cows, but it is likely that fever was a consequence of sensitization to the endotoxin.

This example of sensitization contrasts with the unresponsiveness which was induced by repeated intramammary infusions of low doses of endotoxin for six consecutive milkings (Shuster and Harmon, 1990). Designed to mimic a chronic inflammation, this procedure rendered the cows refractory to the systemic reactions (fever and cortisol increases in blood) provoked by the first infusions. A high initial dose of 100 µg of endotoxin was also reported to provoke unresponsiveness, as measured by the production of TNF-α in milk, following a second infusion of endotoxin into the same quarters (Lahti et al., 1994). To account for these contrasting results, it can be hypothesized that, depending on the quantity of endotoxin responsible for the initial inflammatory reaction, a state of hyper-responsiveness or of hypo-responsiveness can be induced in the mammary gland.

The contribution of TNF-α to the triggering of inflammation and to the innate defenses of the host against infectious agents is well documented (Dinarello, 1983; Van Miert, 1991). On the other hand, an excess of TNF-α production could result in an exaggerated inflammatory response leading to local tissue destruction or even the death of the cow during acute E. coli mastitis (Sordillo and Peel, 1992). The regulation of the synthesis of TNF-α is consequently of utmost importance and from a teleological point of view, it is tempting to assume that mechanisms able to either augment or reduce the response to a given amount of endotoxin play an important role during coliform mastitis.

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