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Short communication

Effects on adhesion molecule expression and lymphocytes in the bovine mammary gland following intra-mammary immunisation

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ABSTRACT

Changes to adhesion molecule expression and lymphocyte populations were evaluated in alveolar mammary tissue collected from cows following an immunisation protocol that involved intra-mammary inoculation to induce an IgA response in mammary secretions. The right quarters of the udder were immunised; the left side acted as a control. Antibody titres in secretions showed that at least two animals responded with antigen-specific IgA. Numbers of T-lymphocytes were 4-fold higher in immunised glands compared with controls ($P < 0.05$). IgA-, IgM- and IgG-positive cell numbers were significantly higher ($P < 0.01$) in immunised glands compared with controls in three of the four cows. No mucosal addressin molecule-1 (MAdCAM-1), vascular cell-adhesion molecule-1 (VCAM-1) or peripheral node addressin (PNAd) protein expression was detected on smaller venules that stained positively for von Willebrand factor in alveolar mammary tissues, from either immunised or control glands. Both VCAM-1 and PNAd were detected on smaller venules in supramammary lymph nodes, however, there was no significant difference between immunised and control glands. Quantification of MAdCAM-1 mRNA showed very low expression in both immunised and control alveolar tissue compared with Peyer's patch positive-control tissue. These findings suggest that the bovine mammary gland is capable of a mucosal antibody response; however, MAdCAM-1 is not involved with lymphocyte homing to the mammary gland in this species.

1. Introduction

The mammary gland requires lymphocytes not only for immune protection from invading pathogens but also to generate antibodies that provide crucial passive immunity to the neonate (Butler, 1994). Immunoglobulins in colostrum and milk are either transported into the gland via the blood, or produced locally in the gland. Local antibody production in the mammary gland requires immune cells in the same locality (Roux et al., 1977) recruited by activated adhesion molecules and/or chemokines (Kunkel and Butcher, 2002). Trafficking of these cells is not a random process and regional specificity is regulated by specific sets of cell-surface adhesion molecules on lymphocytes and complementary receptors expressed on the vascular endothelium of target tissues (Butcher et al., 1999; Picker, 1994; Radi et al., 2001).

In mice the migration of T cells and B cells into mammary tissue changes during pregnancy and with different phases of lactation, with corresponding changes to vascular addressins (Finke and Acha-Orbea, 2001; Tanneau et al., 1999). However, the ruminant mammary gland appears to differ from the mouse in this respect. In an earlier study, we described the expression of vascular addressins in the ruminant mammary gland at four

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Abbreviations: MAdCAM-1, mucosal addressin cell-adhesion molecule-1; PNAd, peripheral node addressin; VCAM-1, vascular cell-adhesion molecule; vWF, von Willebrand factor; IMM, intra-mammary.

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physiological stages (pregnancy, colostrum secretion, lactation and involution) in healthy, untreated animals (Hodgkinson et al., 2007b). Protein expression of vascular cell-adhesion molecule-1 (VCAM-1) and peripheral node addressin (PNAd) was similar to other species. However, no mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) protein expression was observed, in marked contrast to findings in the mouse.

It has been suggested that the gut-mammary axis, where lymphocytes traffic between mucosal inductor and effector sites, may be under-developed in the ruminant (Watson and Kennedy, 1981). A review by Kehrli and Harp (2001) concluded that the ruminant mammary immune system is not closely linked to the intestinal immune system, and therefore, is not part of the common mucosal immune system as defined for mono-gastrics. This was based on reports that intestinal vaccination of ruminants does not appear to induce protective antibodies in milk (Chang et al., 1981; Moon and McDonald, 1983), and on adoptive transfer studies of lymphocytes derived from mucosal and peripheral lymph nodes (Harp and Moon, 1987; Harp et al., 1988). Therefore, it is feasible that the cow does not have, or does not activate the homing mechanisms, including the mucosal addressin, MAdCAM-1, required to attract lymphocytes of mucosal origin to the mammary gland. The observation that IgA is a minor immunoglobulin in ruminant mammary secretions would support this suggestion. However, other reports in the literature suggest that the IgA system in the ruminant mammary gland is naturally dormant but may be stimulated by local antigenic challenge (Lascelles and McDowell, 1970; Lee and Lascelles, 1970).

In this study, we characterised changes in the bovine mammary gland following local immunisation to induce an IgA response in mammary secretions, using an immunisation regimen based on previous work (Hodgkinson and Hodgkinson, 2003). Only one side of the udder received intra-mammary (IMM) immunisation to allow for within-animal comparison of the expression of adhesion molecules between the locally stimulated and non-stimulated glands of each animal. In addition to the expression of MAdCAM-1, VCAM-1 and PNAd, the changes to lymphocyte populations in mammary alveolar tissues were examined.

2. Materials and methods

2.1. Animals

Four pregnant, non-lactating, multi-parous dairy cows of mixed age and breed (Friesian and Jersey) were immunised using an emulsion of *Candida* (*C.*) *albicans* (strain ATCC10231; American Type Culture Collection, Manassas, VA, US) cell suspension (0.5 g protein/l) in one part sterile saline solution (0.9%, Baxter Healthcare, NSW, Australia) with three parts Incomplete Freund's Adjuvant (Sigma Chemical Co., St. Louis, MO, USA), as described previously (Hodgkinson et al., 2007a). Briefly, the procedure involved intra-peritoneal (IP) injections (4 ml) into the peritoneal cavity on the left-hand side of the animal and intra-muscular (IM) injections (2 ml/side) into the anterior neck muscle. IP and IM immunisations were administered at twelve and eight weeks prior to expected calving dates. In addition, IMM immunisations were administered via the teat orifice (2 ml/quarter) at eight and six weeks prior to expected calving dates. Only the two quarters of the right side of the udder were immunised. The left side of the udder acted as a control. All animal treatments were approved by the Ruakura Animal Ethics Committee.

2.2. Sample collection and preparation

The cows were sacrificed seven days following the 2nd IMM immunisation and mammary glands excised. Alveolar tissue samples and supramammary lymph nodes were collected from both the immunised side of the udder and the control side. Peyer's patch tissues were also obtained. Tissues for immunohistochemistry analysis were embedded in O.C.T. (Optimal Cutting Temperature) compound (Tissue Tek, Bayer Diagnostics, Auckland, NZ) snap-frozen in liquid nitrogen and stored at −80 °C or washed (24 h, 4 °C) in phosphate buffered saline (PBS; 10 mM NaH_2PO_4/Na_2HPO_4, 150 mM NaCl, pH 7.4) prior to fixing in 95% ethanol and embedding in paraffin. Tissues for RNA analysis were wrapped in aluminium foil, snap-frozen in liquid nitrogen and stored at −80 °C. Samples of (dry) mammary secretions were collected from the immunised and control quarters on the day prior to slaughter. Samples were centrifuged (1650 × *g*, 10 min, 4 °C), and the fat layer removed. Skimmed samples were then diluted (20%, v/v) with PBS and recentrifuged (11,600 × *g*, 1 h, 4 °C). Supernatants were retained at −20 °C for analysis.

2.3. Specific IgA in mammary secretions

Anti-*C. albicans* IgA titres were measured using a non-competitive indirect ELISA as described previously (Hodgkinson et al., 1995). Briefly, microtitre plates (Maxisorb F-96, Nalge Nunc International, DK-4000, Roskilde, Denmark) were coated with a suspension of the *C. albicans* preparation used for vaccination (100 μl; 10 mg protein/l) in bicarbonate buffer (50 mM $NaHCO_3$, pH 9.8). Test samples and reference sample were assayed at dilutions of 1:100 to 1:100,000. Secondary antibody was rabbit anti-bovine IgA (1:40,000; Bethyl Laboratories). Tertiary antibody was goat anti-rabbit immunoglobulin conjugated with horse-radish peroxidase (HRP) (1:12,000; DakoCytomation, DK-2600, Glostrup, Denmark). Detection substrate contained 0.45 g/l H_2O_2 and 0.1 g/l 3,3′,5,5′-tetramethylbenzidine in acetate buffer (100 mM CH_3COONa, pH 5.4). Optical density (OD) was measured at 450 nm (Plate reader, ELP-35, Bio-Tek). Antibody titres for test samples were defined as the reciprocal of the dilution which produced an OD equal to 50% of the maximum OD above background of the reference sample. Results are expressed as titre units of antibody.

2.4. Immunohistochemistry

For detection of vascular addressins and lymphocyte subsets, serial sections from frozen tissues, 6 μm thick,