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Ann. Rech. Vét., 1983, **14** *(3)*, 271-279

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Résumé

CINÉTIQUE DES CONCENTRATIONS DE SÉRUMALBUMINE, IMMUNOGLOBULINES (IgG1, IgG2, IgM) ET LACTOFERRINE DANS LE LAIT APRÈS L'INOCULATION AVEC *E. COLI* DE LA MAMELLE DE VACHES IMMUNISÉES ET NON-IMMUNISÉES. — Deux vaches immunisées et trois vaches non-immunisées ont été infectées dans un quartier avec la souche vaccinale de *E. coli* (10^4 CFU). L'immunisation a consisté en une injection sous-cutanée au tarissement de bactéries tuées suivie d'un rappel intramammaire cinq semaines plus tard. Les concentrations d'immunoglobulines (IgG1, IgG2, IgM, sérum-albumine (SAB) et de lactoferrine ont été déterminées dans des échantillons de lait prélevés à deux heures d'intervalle pendant les 16 premières heures suivant l'inoculation, puis à chaque traite pendant quatre jours. Immunisés ou non, les quartiers ont commencé à réagir 10 h après l'inoculation. Au début de la phase inflammatoire aigue, les IgG1 et les IgG2 sont passées du sang vers le lait similairement à la SAB. Par la suite, les concentrations d'IgM (et à un moindre degré d'IgG) ont été supérieures à celles attendues sur la base d'un transfert passif. Une exsudation marquée des protéines dosées a été observée pour toutes les vaches sauf une. Chez celle-ci (immunisée), la réaction cellulaire fut cependant intense. Les concentrations de lactoferrine ont augmenté à partir de 24-32 h après l'inoculation et sont restées élevées jusqu'à la fin de la période d'observation dans les quartiers inoculés des vaches non-immunisées, au contraire des quartiers inoculés des vaches immunisées où les concentrations sont restées faibles.

The course of a microbial infection may be studied through the reactions of the host and the changes in the bacterial population *in vivo*. Technical difficulties may preclude or hinder the measurement of these parameters, as occurs, for example, in infection of the skin (McClelland and Van Furth, 1976). Attempts to gain a full understanding of the infective process of bovine mastitis rely mainly on examination of mastitic milk. It has been stated that "staphylococcal mastitis is essentially a duct disease" (Anderson, 1982). This is most likely to apply to *E. coli* mastitis, since even attachment of these bacteria to epithelial cells has not been evidenced (Frost *et al.*, 1980). Thus, at least during the first phase of inflammation, decisive events take place in milk, which is the vehicle of the host defence mechanisms and the "tissue" colonised by bacterial invaders. The ease with which large amounts of inflammatory fluid can be obtained during mastitis give an almost unique opportunity to study in detail the conflicting interactions of pathogens and host defences. Coliform mastitis, which exhibits intense inflammatory reactions, has been the subject of extensive studies using milk samples withdrawn at different times during the course of infection. These studies include relative distribution (Bortree *et al.*, 1962 ; Carroll *et al.*, 1963) or concentrations (Harmon *et al.*, 1976) of whey proteins, and pattern of changes *in vivo* in bacterial population

in relation with phagocytic cells (Schalm *et al.*, 1967; Hill *et al.*, 1978).

The purpose of the present investigation was to determine the pattern of changes in whey proteins following challenge exposure with *E. coli*. Several factors related to specific and non-specific defences were taken into account: immunoglobulins (IgG1, IgG2, IgM), lactoferrin, heat-labile bactericidal activity (complement), and serum albumin (BSA) as a marker of passive exudation. Particular attention was paid to the early phase of the inflammatory response. The influence of immunisation on these events was also investigated.

Materials and Methods

Animals and experimental design

Five healthy Friesian cows were selected on the basis of absence of udder infection at drying-off. Two cows were immunised with an *Escherichia coli* vaccine, the other three cows serving as unimmunised control. The five cows were challenged in a single mammary gland with the vaccine strain about 60 days after calving. In this purpose bacteria (strain B117) were grown overnight, washed, and 1 ml of a bacterial suspension (10^4 CFU/ml) in pyrogen free saline was infused through the teat canal immediately after morning milking. Inoculated glands had foremilk cell-counts below 250 000 cells/ml and were free of infection. Immunised cows were infused in a locally-immunised quarter. The infused quarters were sampled at 2 h-intervals for the first 16 h post-inoculation, then at each milking for at least four days. Cows were milked every day at 7:30 and 16:30.

Vaccine preparation

The *E. coli* strain used for vaccine production was the strain B117 (O80 K85 K99) which has been extensively studied in relation to mastitis (Hill *et al.*, 1978, 1979). Bacteria were grown for 18 h at 37° C in a semi-defined liquid medium (Hill *et al.*, 1976) on a rotary shaker. The bacterial suspension was concentrated using a hollow fibre concentrator with 100 000 MW cut-off (H1P100, Amicon Corporation, Danvers), and washed with distilled water. The final product was centrifuged at 10 000 *g* for 20 min and freeze-dried. Before use bacteria were killed by incubation with 2 % glutaraldehyde (V/V; Serva Feinbiochemica, Heidelberg) for 2 h at room temperature, and washed three times with saline.

Immunisation procedure

Two cows (412 and 607) received a subcutaneous injection containing 5 mg (dry weight) of killed bacteria (B117) in adjuvant consisting of a combination of aluminium hydroxide gel and incomplete Freund's adjuvant (Wells *et al.*, 1979). The bacterial cells were suspended in 0.5 ml of sterile isotonic saline and absorbed onto 1 ml of an aluminium hydroxide gel (Alu Gel S, Serva). The suspension was emulsified in 1.5 ml of incomplete Freund's adjuvant (Difco Laboratories, Detroit). A booster, consisting of 5 mg of killed bacterial cells suspended in sterile isotonic saline, was given intramammarily through the papillary duct five weeks later in two quarters of each cow. The dry period lasted about two months.

Preparation of immunoglobulins

Bovine IgG1 was prepared from colostrum as described elsewhere (Caffin *et al.*, 1983).

To obtain IgM, colostral whey was prepared with rennin and precipitated with ammonium sulphate at 33 % saturation. Following centrifugation the pellet was resuspended in ammonium bicarbonate 0.1 M and applied to Sepharose 6B-CL (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in the same buffer. The IgM fraction was applied to a Sephadex G 25 column (Pharmacia), eluted with 0.03 M phosphate buffer pH 7.3, and fractionated stepwise on a cellulose anion exchange column (DEAE-cellulose, Serva) equilibrated in the same buffer. The IgM fraction was eluted with 0.2 M phosphate buffer pH 6.0, concentrated by ultrafiltration (XM50 membrane, Amicon), filtered through, Sepharose 6B-CL and freeze-dried. This preparation gave only one precipitating line in double diffusion in gel and immunoelectrophoresis against rabbit anti-bovine whole serum and anti-colostral whey antisera, and reacted with specific anti-bovine IgM (Miles Laboratories, Paris France).

Bovine IgG2 was prepared from pooled serum. Following precipitation with ammonium sulphate at 33 % saturation, the pellet was resuspended in 0.01 M phosphate buffer pH 8.0 and desalted on a Sephadex G-25 column. The immunoglobulin fraction was applied to a cellulose anion exchange column (DEAE cellulose, Serva) equilibrated in the same buffer, according to Levieux (1974). The fall-through peak was filtered through Sepharose 6B-CL equilibrated in 0.1 M ammonium bicarbonate and freeze-dried. Purity was checked by double diffusion in gel and immunoelectrophoresis against specific anti-bovine IgG2 (kindly provided by D. Levieux) and against rabbit anti-bovine whole-serum antiserum.

Preparation of specific antisera

The method of Binaghi *et al.* (1967) was used. Guinea-pigs received two injections consisting of 0.3 mg IgM or 0.15 mg IgG2 emulsified in complete Freund's adjuvant (Difco). Guinea-pig anti-bovine IgG2 sera were rendered subclass-specific by absorption on Sepharose 4B-bovine

Table 1. — Whey protein levels in inoculated quarters the day before challenge (about two months after calving).

Whey proteins (mg/ml)	Immunised cows		Unimmunised cows		
	412	607	002	004	007
Serum/albumin	0.25	0.25	0.20	0.16	0.25
IgG1	0.18	0.38	0.26	0.15	0.16
IgG2	0.02	0.02	0.02	0.02	0.02
IgM	0.17	0.14	0.18	0.20	0.14
Lactoferrin	0.04	0.06	0.03	0.03	0.04

Table 2. — Concentrations of Igs and serum/albumin in blood serum of cows the day before intramammary challenge.

Blood proteins (mg/ml)	Immunised cows		Unimmunised cows		
	412	607	002	004	007
Serum/albumin	36.5	40	40	38	42
IgG1	12.8	16.5	12.4	11.6	12.4
IgG2	11.1	14.2	8.2	9.0	9.3
IgM	2.8	3.1	2.6	3.5	3.0

IgG1. Antibodies to α 2 macroglobulin were removed from anti-bovine IgM by absorption on Sepharose 4B - bovine α 2 macroglobulin. Isolation of α 2 macroglobulin was performed by fractionation of fœtal calf serum (Flow Laboratories, Asnières France) on Sepharose 6B-CL. Immunosorbents were prepared according to Porath *et al.* (1967). Specificity of antisera was checked by immunoelectrophoresis and double gel diffusion against bovine serum and colostral whey.

Sample preparation

Skim milk samples (cell-free milk) were prepared by centrifuging milk at 1 000 *g* for 20 min to remove cells and fat, then at 3 000 *g* for 30 min to remove bacteria. Whey was obtained by incubation at 37° C for 1 h with rennin and centrifugation (10 000 *g* for 20 min). Blood was obtained by tail vein puncture, allowed to clot at room temperature, and sera prepared by centrifugation at 1 000 *g* for 20 min. Cell-free milk, whey, and serum samples were stored at - 20° C until used.

Protein quantitation

Protein concentrations in whey and serum were determined by the single radial immunodiffusion method of Mancini *et al.* (1965). Every sample was tested in duplicate and the mean used in determining concentration.

Bovine serum albumin, lactoferrin, and their specific antisera were prepared as described elsewhere (Poutrel *et al.*, 1982; Rainard *et al.*, 1982). Protein concentration of standard solutions was estimated assuming extinction cœfficients (absorbancy at 280 nm per mg per ml) of: BSA, 0.66 (Sober, 1970); IgG and IgM, 1.37 (Duncan *et al.*, 1972); lactoferrin, 1.45 (Castellino *et al.*, 1970).

Somatic cell-counting

Somatic cell count was determined with a Coulter Counter (Model F, Coultronics, Margency France) according to the procedure recommended by the International Dairy Federation (1979).

Assessment of cell-free milk activities against strain B41

The serum-sensitive test organism *E. coli* strain B41 (O101 K99) and the microtiter plate method described previously were used (Rainard, 1983). Briefly, about 5×10^2 colony-forming units (CFU) in saline (20 µl) were added to round-bottom microtiter wells with 80 µl of cell-free milk. Bacterial viable counts (pour-plate technique) were made before and after incubation at 37° C for

2 h 30. Every sample, was tested in duplicate and heat-sensitivity was checked by heating one part at 56° C for 30 min before testing. Milk was considered bactericidal if less than 20 % of the initial bacterial count was recovered after incubation. Bacteriostasis was assumed to occur when bacterial count after incubation was less than twice the initial count, as substantiated in previous experiments (Rainard, 1983).

Results

Whey protein levels before challenge exposure

Table 1 shows that the immunisation procedure did not cause a noticeable increase in whey protein levels at time of challenge exposure. In particular BSA concentrations were similar in immunised and unimmunised animals, suggesting that immunised quarters were uninflamed at that time.

Whey protein-changes during inflammation

For all the cows exudation of blood serum proteins started after 8 h postinoculation (1st milking after inoculation) and before 10 h postinoculation (fig. 1-5). The most significant feature of the whey protein variations was that albumin, IgG1 and IgG2 rose in parallel and decreased together. A selective index was calculated to compare transfers of Igs and BSA, with correction for differences in their respective concentrations in blood serum. Serum values are given in table 2.

selective index =
(lacteal Ig/serum Ig) × (serum BSA/lacteal BSA)

Selective indexes for IgG1, IgG2, and IgM were calculated at the onset of inflammation (10 h postinoculation) and at 72 h postinoculation, when the reaction had begun to subside (table 3). It should be noted that a selective index of 1 can be interpreted as a non-selective transfer from blood into milk. This was the case with IgG1 during the acute phase and IgG2 during the entire inflammatory period. The IgG1 selective index

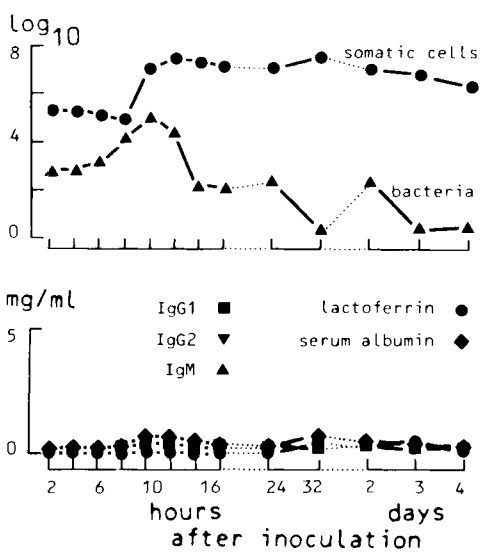

Fig. 1. — Changes in whey protein concentrations, bacterial and somatic cell-counts, in the challenged quarter of cow 412 (immunised) from inoculation (zero time) to four days post-inoculation.

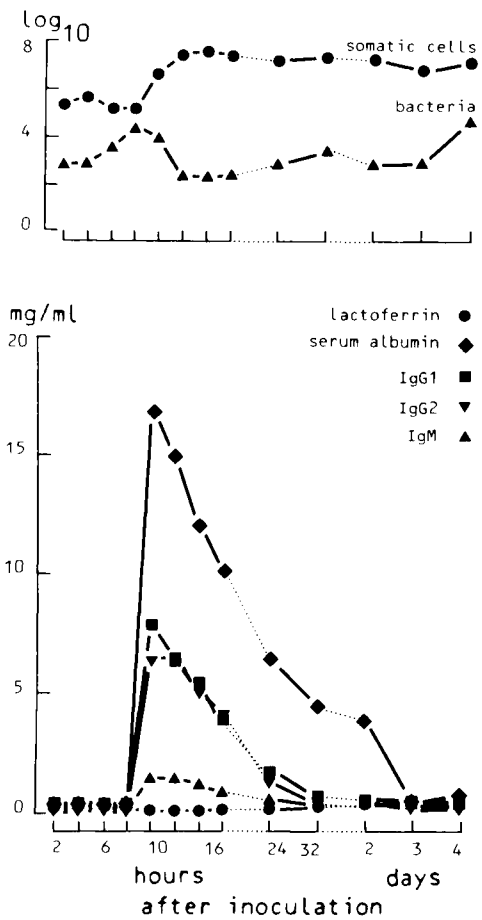

Fig. 2. — Changes in whey protein concentrations, bacterial and somatic cell-counts, in the challenged quarter of cow 607 (immunised) from inoculation (zero time) to four days post-inoculation.

rose slightly when inflammation abated. In fact, only IgM gave an index significantly more than 1, especially when BSA whey concentrations had declined.

An interesting picture was seen with cow 412 (fig. 1): somatic cell-counts rose markedly whereas exudation of blood serum proteins remained limited, giving a striking example of dissociation between the two components of the inflammatory response.

Cow 002 displayed a delayed recruitment of cells and exudation of blood proteins (fig. 3). In fact the reaction started at 10 h post-inoculation, as for the other cows, but was of comparatively lower intensity during the early phase. The bacterial population reached an unexpectedly high size ($> 10^9$ CFU/ml) and the cow suffered from an acute form of mastitis with the associated systemic clinical signs. Despite spontaneous sterilisation, the inoculated quarter remained dry thereafter.

Lactoferrin concentrations did not rise markedly in the whey of the two immunised cows. For the three unimmunised animals, a pronounced increase was noted, starting later and persisting longer than increase in BSA values.

Antibacterial activities of cell-free milk

Cell-free milk exhibited a heat-labile bactericidal activity against the serum-sensitive strain B41 at the onset of the inflammatory reaction. Heat-treated milk samples of cow 412 did not show any antibacterial activity under the limit of the test. Slowing down or prolongation of the lag-phase of

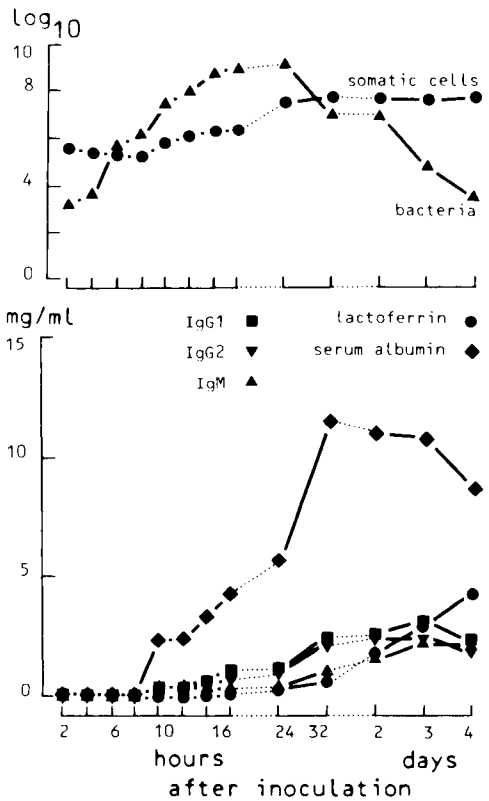

Fig. 3. — Changes in whey protein concentrations, bacterial and somatic cell-counts, in the challenged quarter of cow 002 (unimmunised) from inoculation (zero time) to four days post-inoculation.

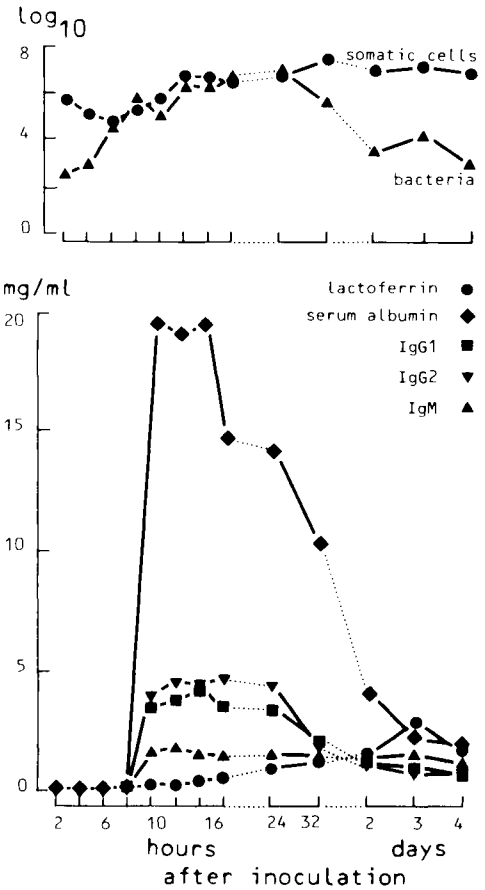

Fig. 4. — Changes in whey protein concentrations, bacterial and somatic cell-counts, in the challenged quarter of cow 004 (unimmunised) from inoculation (zero time) to four days post-inoculation.

Table 3. — Selective indexes of Ig transfer into milk in the early phase (10 h post-inoculation) and late phase (72 h post-inoculation) of inflammation in inoculated quarters.

Hours post-inoculation	Immunoglobulin G1	G2	M
10	0.98	1.01	1.97
72	1.43	0.90	7.60

selective index = (lacteal Ig/Serum Ig) × (Serum BSA/Lacteal BSA).
Data are means of the five inoculated glands (five different animals).

Table 4. — Antibacterial activities of mastitic cell-free milk samples taken at intervals after inoculation on the serum-sensitive strain B41.

	Immunised cows				Unimmunised cows			
	412		607		004		007	
Hours post-inoculation for samples on the test	cell-free milk	heat treated	cell-free milk	heat treated	cell-free milk	heat treated	cell-free milk	heat treated
0	G	G	ST	G	G	G	G	G
8	G	G	ST	G	G	G	G	G
10	G	G	K	ST	K	G	K	ST
12	K	G	K	ST	K	ST	K	ST
14	K	G	K	ST	ND	ND	K	ST
16	K	G	K	ST	ND	ND	K	ST
24	K	G	K	ST	ND	ND	K	ST
32	K	G	K	ST	K	ST	K	ST
48	K	G	K	K	K	ST	K	K
72	K	G	K	K	K	ST	K	K
96	ST	G	K	K	K	ST	K	ST

Cell-free milk samples, either heat-treated (at 56 °C for 30 min) or untreated, were incubated 2.5 h with *E. coli* B41.
G = growth; ST = bacteriostasis; K = killing.
ND = not done (contaminated samples). Most samples from cow 002 remained contaminated with strain B117 after preparation and were not assayed.

bacterial growth occurred for other cows as soon as 10 h or 12 h post-inoculation. This was followed by a heat-resistant killing activity in samples from cows 607 and 007.

Because of the great size of the bacterial population ($> 10^9$ CFU/ml) reached in the inoculated gland of cow 002, several milk samples remained contaminated after preparation. However, a bactericidal activity against *in vitro* grown *E. coli* B 117, assayed as described previously (Rainard, 1983) was found in the 96 h post-inoculation sample and persisted in subsequent samples taken up to six days post-inoculation.

Discussion

In agreement with a previous report on experimental mastitis performed in similar conditions (Rainard, 1983), inflammatory responses were triggered between 8 h and 10 h post-inoculation. Simultaneous increase in cell count, blood protein exudation and appearance of heat-labile bactericidal activity most likely complement-mediated were noted. Concomitant changes in IgG1 and IgG2 concentrations were shown in the present study. This is in contrast with results of Ost *et al.* (1978) who found much higher amounts of IgG2

than would be expected on the basis of serum exudation during the first hours of inflammation following intramammary injection of *E. coli* endotoxin. One possible explanation would be that IgG2, known to be cytophilic for polymorphonuclear leukocytes (McGuire *et al.*, 1979) is conveyed by these cells in mastitic milk. Assuming one IgG molecule occupies one receptor site and one cell bears up to two million receptors (Arend and Mannik, 1975), 2×10^7 cells could adsorb about 11 µg IgG2. This suggests that contribution by leukocytes to IgG2 concentrations in acute mastitis milk is very low.

Protracted persistence of elevated levels of IgG after BSA levels had fallen was not seen (fig. 1-5), in contrast to previous reports (Harmon *et al.*, 1976; Anderson and Andrews, 1977; Ost *et al.*, 1978). Only IgM was found to obey a pattern of transfer different from non-selective exudation, since the selective index for this isotype exceeded

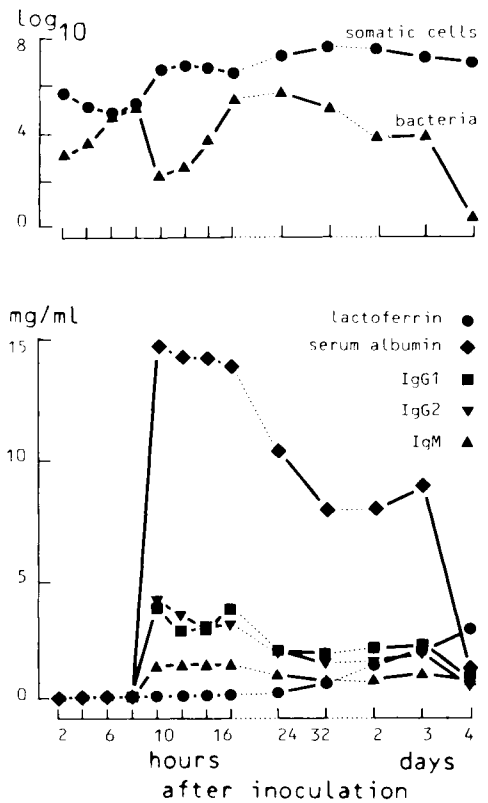

Fig. 5. — Changes in whey protein concentrations, bacterial and somatic cell-counts, in the challenged quarter of cow 007 (unimmunised) from inoculation (zero time) to four days post-inoculation.

one, particularly when the acute phase of inflammation had subsided (table 3). Anderson and Andrews (1977) reported a similar observation applying to IgA and IgM. Local synthesis of antibodies is not likely to account for the amounts of IgM encountered as soon as 72 h after antigenic stimulus. An hypothesis would be that a selective transport, possibly like that described by Brandtzaeg and Baklien (1977), operates in the gland in a stimulated manner following inflammation.

It has been found that the magnitude of lactoferrin changes due to acute mastitis is related to the severity of infection, coliform bacteria causing a greater increase than *Staphylococcus aureus* (Harmon *et al.*, 1975). In a previous experiment on *E. coli* mastitis, lactoferrin changes were slight only in the cow that did not develop clinical mastitis (Rainard, 1983). In the present report, this occurred in the immunised cows, whereas unimmunised animals showed significant rises. This may be interpreted as a reduced stimulation in immunised glands, resulting from a milder form of inflammation.

Cow 002 mobilised sluggishly her defences during the first day post-inoculation. This was accompanied by a burst of bacterial growth. Such a picture may occur in mid-lactation but is more characteristic of cows near calving (Hill *et al.*, 1979). At 24 h post-inoculation, the bacteria-to-cells ratio was 28. Under these circumstances, properties of cell-free milk are likely to be of significance for the outcome of infection. In fact mastitic cell-free secretion of cow 002 acquired a bactericidal activity against the invading strain, as may occur in acute mastitis (Rainard, 1983). Nevertheless, killing of *in vitro* grown bacteria was much more rapid (unpublished results) than the decrease of bacterial population *in vivo* (fig. 3). Bacterial adaptation to intramammary environment may account for this difference, and this point deserves further investigation.

Bacteriostatic activity was detected in many samples of heat-treated cell-free milk (table 3). This activity has been shown to be reversed by iron (Rainard, 1983) and was surmised to be due to iron-binding proteins. In the case of cow 607, which did not exhibit substantial increase in lactoferrin levels, serum transferrin may be responsible for the bacteriostasis that appeared in the course of infection. Nevertheless, it should be noted that in the screening test used in this experiment the incubation time (2.5 h) was insufficient to distinguish between a prolonged lag-phase and a real slowing-down of bacterial growth.

In conclusion, it can be stated that immunisation did not modify noticeably the mode of transfer of blood proteins from blood into milk. Decrease in the magnitude of changes in milk whey protein concentrations, such as blood proteins for

cow 412, and lactoferrin for the two immunised animals, occurred but needs confirmation with more animals. Evidence that influx of cells is not systematically linked to exudation of blood proteins was obtained (cow 412). In this report specific antibody activity of Igs was not taken into account but their opsonising activity, the role of phagocytic cells and the influence of immunisation on severity of the disease were also investigated and are reported in the accompanying paper (Rainard, 1983b).

Accepted for publication, 10 th March 1983.

Acknowledgements

Thanks are due to Dr G. Dubray for vaccine preparation and to J. Dufrenoy for technical assistance with preparation of immunologic reagents.

Summary

Two immunised and three unimmunised cows were infected in a single mammary gland with 10^4 CFU of the vaccine *Escherichia coli* strain. Immunisation comprised systemic (subcutaneous) injection of killed bacteria at drying-off and one intramammary infusion five weeks later. Immunoglobulin (Ig) G1, IgG2, IgM, serum albumin (BSA) and lactoferrin concentrations were monitored by sampling the inoculated glands at 2 h-intervals during the first 16 h post-inoculation, then at each milking for four days. Whether immunised or not, mammary glands started to react at 10 h post-inoculation. During the early acute phase, IgG1 and IgG2 permeated from blood into milk at a rate similar to BSA. Later on, IgM (and at a lower degree IgG1) concentrations were higher than expected on the basis of passive transfer. Marked protein exudation was seen in all of the cows but one. Nevertheless, this cow (immunised) showed an intense cellular reaction like the other animals. Lactoferrin concentrations rose from 24-32 h post-inoculation and remained elevated to the end of the observed period in inoculated quarters of unimmunised cows. By contrast, in immunised cows lactoferrin concentrations remained low. Heat-labile bactericidal activity against a serum-sensitive *E. coli* strain appeared concomitantly with rise in BSA concentration. Heat-resistant bactericidal activity of cell-free milk was detected one or two days later in three of the cows. Bacteriological cure of quarters occurred without therapy in all cases.

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