EXPERIMENTAL MASTITIS WITH ESCHERICHIA COLI: SEQUENTIAL RESPONSE OF LEUKOCYTES AND OPSONIC ACTIVITY IN MILK OF IMMUNISED AND UNIMMUNISED COWS

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Résumé

MAMMITE COLIBACILLAIRE EXPÉRIMENTALE: CINÉTIQUES DE L'AFFLUX DE CELLULES ET DE L'ACTIVITÉ OPSONISANTE DANS LE LAIT DE VACHES IMMUNISÉES OU NON-IMMUNISÉES. — Deux vaches immunisées et trois vaches non-immunisées ont été infectées dans un quartier avec la souche vaccinale de *Escherichia coli* (B117). L'immunisation a consisté en une injection sous-cutanée, au tarissement, de bactéries tuées avec adjuvant et d'un rappel intramammaire (sans adjuvant) cinq semaines plus tard. Les numérations cellulaires et bactériennes dans le lait ont été enregistrées tout au long de l'expérience. Les propriétés bactéricides et opsonisantes du lait ont été évaluées avant l'inoculation d'épreuve et 6, 12 et 24 h après l'inoculation. Avant l'épreuve, le lait acellulaire des vaches immunisées a permis aux polynucléaires du sang de tuer les bactéries de la souche vaccinale, tandis que dans le lait des vaches non-immunisées la taille de la population bactérienne a pu s'accroître. Cependant *in vivo* les bactéries ont été capables de se multiplier dans le lait de toutes les vaches jusqu'à ce qu'elles déclenchent une réaction inflammatoire. L'afflux de cellules débuta entre la huitième et la dixième heure suivant l'inoculation chez tous les animaux, mais fut plus intense chez les vaches immunisées pendant les six premières heures de l'inflammation. Les tests *in vitro* ont montré que le lait de mammite a acquis une forte activité bactéricide au début de l'inflammation. Quoiqu'une des vaches immunisées ait montré des signes cliniques de mammite, la production de lait des animaux non-immunisés chuta de façon plus importante. Ces résultats suggèrent que l'immunisation a permis l'augmentation du recrutement des cellules phagocytaires et l'établissement d'une activité opsonisante dans le lait avant la réaction inflammatoire.

Both the humoral components and the phagocytic cells acting separately or together, are of importance in the removal of pathogenic coliform bacteria from the mammary gland (Schalm *et al.*, 1964a; Jain *et al.*, 1967). Much attention was paid to the elimination of bacteria through phagocytosis, and the conditions for an efficient phagocytic-killing *in vivo* were identified by use of experimental infections: a) leukocytosis has to be present (Schalm *et al.*, 1964b) or has to occur rapidly after infusion of bacteria (Hill, 1979); b) opsonic activity is required in milk to enable polymorphonuclear leukocytes (PMNs) to ingest and kill bacteria (Jain and Jasper, 1967).

In a recent report, studies were devoted to the antibacterial activities of cell-free mastitic milk in the course of the inflammatory response following infection with *E. coli* (Rainard, 1983). By use of a similar experimental procedure, the following study was carried out to determine to what extent immunisation with killed bacterial cells during the dry period would modify the inflammatory response in the course of experimental *E. coli* mastitis.

Materials and Methods

Animals and experimental design

Two cows (412 and 607 in their 7 th and 5 th lactation respectively) were immunised by subcutaneous route at drying-off and boosted intramammarily five weeks later (about three weeks before calving) with the serum-resistant strain *E. coli* B117 (O80 K85 K99). Details are given in the accompanying paper (Rainard and Caffin, 1983). Three other cows (002, 004 and 007), in their first lactation, served as unimmunised controls. The five animals were challenged in a single mammary gland with *E. coli* B117 when they were about 60 days in lactation. 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. Milkings took place at 7:30 and 16:30 every day. Clinical signs and milk production were recorded.

Udder inoculation

This was performed as described previously (Rainard, 1983). Briefly, bacteria (B117) were grown overnight, washed and 1 ml of bacterial suspension (10^4 CFU/ml) in pyrogen-free saline was infused through the teat canal immediately after the morning milking.

Sample preparation

Milk was drawn aseptically and chilled in ice. A portion was used in the whole milk bactericidal assay. The remainder was centrifuged to remove cells and fat (cell-free-milk) and processed as described (Rainard and Caffin, 1983).

Somatic-cell-counting

The procedure recommended by the International Dairy Federation (IDF, 1979) was followed, using a Coulter Counter (Model F, Coultronics).

PMN bactericidal assay

A microtitre plate method was used. Blood PMNs were isolated by the method of Carlson and Kaneko (1973). Two washings of PMNs were done in Dulbecco's phosphate buffer saline (PBS) at room-temperature to eliminate cytophilic immunoglobulins (Williams and Bunch, 1981). Viability was determined by the trypan blue dye exclusion technique and was always greater than 96 %. The same cow was used as a source of PMNs throughout the experiment. The phagocytic mixture consisted of 0.04 ml of PMN suspension (10^7 cells/ml) in PBS supplemented with 0.1 % glucose, 0.05 ml of opsonin source (cell-free milk or diluted serum) and 0.01 ml of *E. coli* B117 suspension (2.4 x 10^7 CFU/ml) in PBS supplemented with 0.1% human serum albumin. The microtitre plate was placed on a vertical turntable at 37 °C and rotated at 30 rpm. Twenty microlitre samples were taken at zero time and 120 min, serial ten-fold dilutions made, and viable counts estimated by the pour-plate technique. The first dilution was performed with vigorous vortexing in distilled water containing 0.5 % sodium deoxycholate to disrupt PMNs. Subsequent dilutions were made in PBS containing 0.1 % human serum albumin.

Assay of bactericidal activity of whole fresh milk

The technique described above was used except that the phagocytic mixture consisted of 0.09 ml whole fresh milk (used within 4 h after collection) and 0.01 of bacterial suspension (*E. coli* B117, 10^6 CFU/ml).

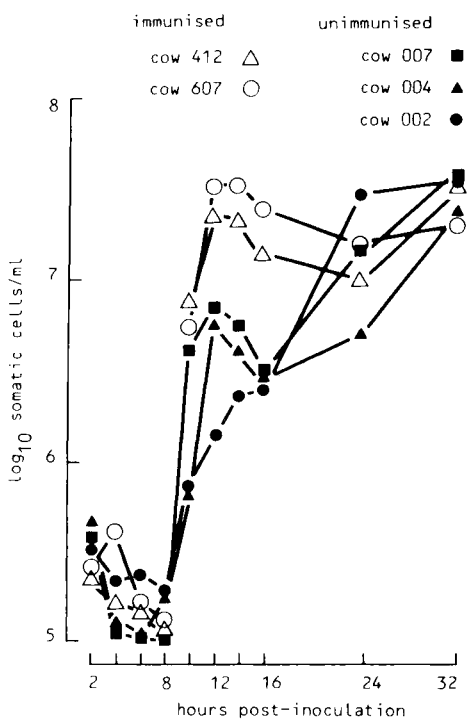

Fig. 1 — Time-course of the influx of somatic cells in the inoculated mammary glands of immunised and unimmunised cows following challenge at zero time with 10^4 CFU/ml of *E coli* B117.

Results

Influx of somatic cells (fig.1)

In all cases, somatic cell counts increased suddenly just before 10 h post-inoculation. At this time, two groups of cows could be distinguished on the basis of the magnitude of cell response: cows 412, 607 and 007 on the one hand, and cows 004 and 002 on the other hand, the latter group with a much lower response. From 10 h post-inoculation till 16 h post-inoculation, the immunised cows (412 and 607) had higher cell counts than the three unimmunised cows. At 32 h post-inoculation, all of the cows exhibited similar somatic cell counts. It is worth noting that the cell response of cow 002 also began at 10 h post-inoculation but was strikingly reduced in magnitude in comparison with the other animals.

Opsonic activity of blood serum

PMNs failed to kill *E. coli* B117 when incubated in PBS. Presence of opsonins in sera of the cows under test was assessed a few days before inoculation. When incubated with 5% blood serum (final concentration) of any cow, whether immunised or not, PMNs killed more than 99.8% of bacteria (PMN-to-bacteria ratio: 1.5:1).

Effect of immunisation on opsonic activity of normal milk (fig. 2)

Opsonic activity of cell-free milk withdrawn before inoculation was assessed in the PMN bactericidal assay. There was a clear-cut difference: the result was a reduction in bacterial population in milk of immunised cows and bacterial growth in milk of unimmunised cows. However, incubation of milk and bacteria without blood PMNs suggested that a slight phagocytosis could have occurred for unimmunised animals. Apparently, growth did not take place in cell-free milk of immunised cows, but it should be noted that an incubation time of 2 h does not permit a precise assessment of bacteriostasis.

Kinetics of opsonophagocytic activity of milk in the course of the inflammatory reaction

— Activity of whole fresh milk (table 1)

Milk was not bactericidal at 6 h post-inoculation. As expected, it became highly bactericidal at the onset of the inflammatory response. This activity declined slightly at 24 h post-inoculation (cows 412, 607 and 007). For cow 004, the overall result of *in vitro* incubation of the 24 h post-inoculation sample was a further increase in bacterial population.

— Activity of cell-free milk with blood PMNs (table 2)

Opsonic activity appeared (unimmunised cows) or was reinforced (immunised cows) in 12 h post-inoculation milk — (10 h post-inoculation samples were not assayed). It should be noticed that activity of the 12 h post-inoculation sample of cow 004 was much lower than that of other cows. Unfortunately, the 24 h post-inoculation sample was not usable, nor cow 002 samples.

Severity of the disease

Clinical signs were recorded and their severity paralleled fairly well the decrease in milk production. Milk yields are shown in table 3. Cow 412 did not display clinical signs at any time. Cows 607 and 007 exhibited an intermediate degree of mastitis, whereas severe mastitis developed for the other two unimmunised cows. The inoculated

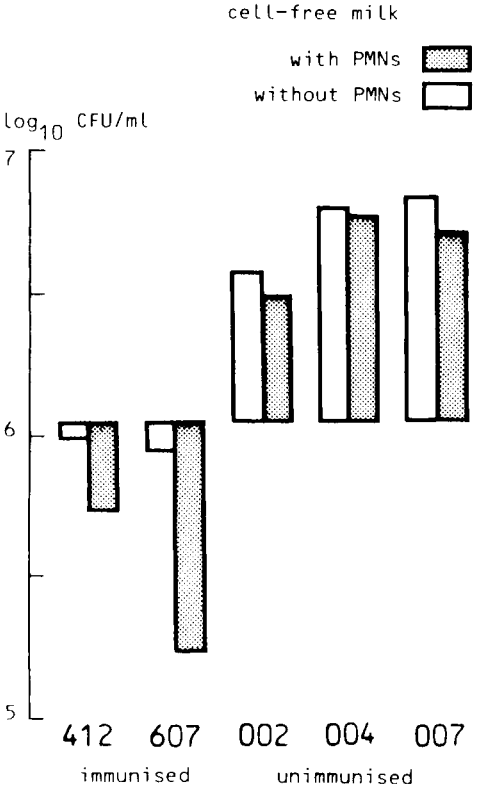

Fig. 2 — Opsonic activity of cell-free milk from the inoculated glands the day before challenge. Blood PMNs and *E. coli* B117 were incubated for 2 h at 37 °C at a PMN-to-bacteria ratio of 1 : 1 with 50% cell-free milk as the opsonin source. Bacterial growth was assessed by incubating *E. coli* without PMNs.

quarter of cow 002 remained dry during the rest of lactation.

Discussion

Enhanced antibody activity in bovine milk has been shown following local vaccination with an *E. coli* vaccine (Wilson, 1972). However, vaccination (by systemic route) of a cow with a coliform (*Aerobacter aerogenes*) was reported not to increase the opsonic activity of normal milk (Jain *et al.*, 1967). Nevertheless, some authors have reported increase in opsonic activity of milk against *Staphylococcus aureus* by use of systemic immunisation with a live vaccine in sheep (Watson, 1975) or intramammary vaccination with a killed vaccine in cattle (Guidry *et al.*, 1980b). The results presented here evidenced that, more than two months after calving, skim-milk from immunised glands supported phagocy-

Table 1. — Survival of *E. coli* B117 following incubation at 37° C for two hours in whole milk withdrawn at 6, 12 and 24 h post-inoculation with the addition of about 10^5 *in vitro* grown bacteria

Hours post-inoculation	Immunised cows				Unimmunised cows					
	412		607		007		004		002	
	CFU/ml x 10^5[a]	% survival	CFU/ml x 10^5	% survival	CFU/ml x 10^5	% survival	CFU/ml x 10^5	% survival	CFU/ml x 10^5	% survival
6	1.11	126	1.13	111	1.17	391	1.15	452	2.1	381
12	0.92	0.002	0.91	0.001	0.94	0.003	22.9	0.032	10^3	ND[b]
24	0.78	20	0.76	1.8	4.75	0.006	81	185	10^4	ND

a: No. of bacteria before incubation *in vitro* + *in vitro* grown bacteria
b: not determined

Table 2. — Survival of *E. coli* B117 after two hours incubation with blood PMNs in cell-free milk withdrawn at 6, 12 and 24 h post-inoculation.

Hours post inoculation	Immunised cows		Unimmunised cows		
	412	607	007	004	002
6	48	18.6	330	440	310
12	0.23	0.21	0.25	9.68	ND
24	13.8	1.65	0.35	ND	ND

In vitro grown bacteria (2-4 x 10^6 CFU/ml) were incubated for two hours at 37° C with blood PMNs (4 x 10^6 cells/ml) in cell free milk as a source of opsonin
ND: not done

Table 3. — Decrease in milk production of cows challenged on day 0 with *E. coli* B117

Days	Immunised cows		Unimmunised cows		
	412	607	007	004	002
− 7	27.0[a]	24.0	18.4	24.0	17.8
+ 1	17.0	12.4	9.0	3.2	0.2
+ 2	21.4	15.0	10.6	10.0	1.2
+ 7	21.2	20.8	17.7	18.0	12.0
+ 14	23.0	22.2	16.4	18.8	13.4

a = milk yield (kg/day)

tosis of *E. coli* much better than milk from non-immunised animals. This was not achieved through exudation of serum proteins due to inflammation since serum albumin values did not augment in milk of immunised uninflamed quarters (Rainard and Caffin, 1983). Watson and Lascelles (1975) demonstrated that a single systemic administration of antigen with adjuvant during mammary involution induced high titres of specific antibody in milk in the subsequent lactation. Even higher titres were obtained by conjunction of systemic and local immunisation, and it was this procedure that was retained in the present study. The fact that these antibodies belonged mainly to the IgA and IgM isotypes in particularly attractive since it has recently been shown that IgM possess opsonic activity in cattle (Williams and Hill, 1982).

The testing of antibacterial activity of whole milk showed that, during inflammation, opsonophagocytic killing occurred in all glands tested whether immunised or not. Thus the usefulness of preexisting opsonic activity in normal milk is questionable when concomitant influxes of cells and opsonins develop through inflammation as normally occurs in *E. coli* mastitis. Nevertheless, a dissociation between these two phenomena is possible, i.e. recruitment of cells without marked exudation (Rainard and Caffin, 1983) and in such a case preexisting opsonising activity may be of significance.

In this study, all of the cows possessed high amounts of opsonins in serum. These opsonins are presumably opsonising antibodies, since there is no absolute requirement for complement for opsonisation of *E. coli* by serum of adult cow (Williams and Hill, 1982). In fact, «non-immune» cows were not available in the experimental herd, although *E. coli* B117 had not been used before and was reported to have high requirements for opsonisation (Hill, 1981). Existence of high amounts of serum opsonins to the test organism may explain the discrepancy between the results of Guidry *et al.* (1980a), who found that inflammation did not increase opsonisation by milk, and the results obtained in the present experiment, and by Jain and Lasmanis (1978), who found that mastitic milk had higher opsonic activity than normal milk.

The considerable *in vitro* bactericidal activity of whole milk in the early inflammatory response evidenced that freshly recruited phagocytic cells can efficiently operate in milk, despite the detrimental effects of casein and fat on phagocytic killing (Russel and Reiter, 1975; Paape and Guidry, 1977).

In similar conditions Hill *et al.* (1978) showed that mastitic milk could kill added *in vitro* grown bacteria after removal from the gland. In this respect the 12 h post-inoculation sample of cow 004 had lower bactericidal (whole milk) and opsonic (cell-free milk with blood PMNs) activities than the three cows that did not develop very severe mastitis. As for cow 002 bacterial growth *in vivo* was not successfully hindered. As in a previous report (Rainard, 1983), the ability of the gland to preclude the size of the bacterial population reaching levels of $10^6 - 10^7$ CFU/ml in the early hours of infection seemed to be decisive for the course and severity of the disease.

An interesting observation was the different influx of cells in immunised and unimmunised animals: the lag-phase was not shortened but the magnitude was increased in immunised quarters. A recent paper of Colditz and Watson (1982) reported that intramammary immunisation of ewes enhanced the neutrophil influx without modifiying the time of initial cell response. Similarly, Targowski and Berman (1975) reported that lactating glands displayed higher cell counts following infusion of staphylococcal cell walls when they were previously vaccinated with this antigenic material. Immune-mediated inflammation has been demonstrated in the lumen of mammary glands of cows immunised (systemic route) with ovalbumin (De Cueninck *et al.*, 1979). Moreover the speed and magnitude of mobilisation of neutrophils after infection with *E. coli* has been shown to be an important factor in determining the outcome of mastitis (Hill, 1981). Thus it is suggested that immunisation may protect the mammary gland not only by eliciting opsonic antibodies but also *via* enhancement of neutrophil recruitment during the early phase of the inflammatory response.

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Summary

Two immunised and three unimmunised cows were challenged in a single mammary gland with 10^4 colony forming units of the vaccinal *Escherichia coli* strain. Immunisation comprised subcutaneous injection of killed bacteria with adjuvant at drying-off, and one intramammary infusion (without adjuvant) five weeks later. Somatic-cell counts and bacterial counts were monitored throughout the experiment. Bactericidal and opsonic properties of milk were assessed before inoculation and at 6, 12 and 24 h post-inoculation. Before challenge, cell-free milk of immunised cows enabled blood PMN leukocytes to kill the *E. coli* vaccine strain (B117) whereas in cell-free milk of unimmunised cows growth resulted. Nevertheless, *in vivo E. coli* B117 were able to grow in milk of all of the cows until they triggered an inflamma-

tory reaction. Influx of cells started between 8 and 10 h post-inoculation in all of the cows but was more intense in immunised cows during the first six hours of inflammation. *In vitro* tests showed that whole mastitic milk acquired high bactericidal activity at the onset of inflammation. Although one immunised cow displayed clinical signs, milk yields of unimmunised animals were depressed to a higher extent. These results suggest that immunisation was able to enhance recruitment of phagocytic cells and to establish pre-inflammatory opsonic activity in milk.

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