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# Complement factor B and the alternative pathway of complement activation in bovine milk

By PASCAL RAINARD*

*Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, 37380 Nouzilly, France*

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Summary. The contribution of the alternative pathway of complement activation to the capacity of normal milk to deposit C3 fragments on bacteria was tested by attempting to block C3 deposition with antibodies to the alternative pathway component factor B (fB). Factor B was purified and antibodies of the IgY class, which does not activate mammalian complement, were obtained from the egg yolk of immunized laying hens. These antibodies specifically inhibited the deposition of C3. This inhibition and the absence of deposition of C4 demonstrated that C3 deposition in normal milk resulted from the activation of the alternative pathway. Antibodies raised in rabbit were used to develop an ELISA for measuring fB concentrations in milk. The mean concentration of fB was 2·06 $\mu$g/ml ($\pm$0·18, SEM), 0·57 % of the mean value found in serum (360 $\mu$g/ml). This proportion was comparable to that of serum albumin (0·63 % of serum value) but less than the proportion of C3 in milk (2·71 %). Nevertheless, fB was apparently not a limiting factor for the functioning of the alternative pathway, since addition of purified fB to normal milk did not improve C3 deposition. In serum, mild heat-treatment (56 °C for 3 min or 50 °C for 45 min) blocked the alternative pathway and destroyed fB, as shown by loss of antigenicity in ELISA. In milk, mild heat-treatment did not abrogate C3 deposition, and fB was protected, retaining its functionality and antigenicity. Heating at 56 °C for at least 45 min was necessary to completely inhibit C3 deposition in normal milk.

Keywords: Complement, milk, cattle, mastitis, opsonization.

Normal bovine milk (from uninfected, uninflamed glands) lacks haemolytic activity and consequently has long been considered virtually devoid of complement (Mueller *et al*. 1982). Nevertheless, sizeable amounts of C3 have been found in milk (Mueller *et al*. 1982) and more recently deposition of C3 fragments on bacteria has been demonstrated after incubation in normal milk (Rainard & Poutrel, 1995). Opsonization of bacteria by C3 fragments is one of the major effector functions of the complement system. By favouring phagocytosis of bacteria by neutrophils, milk complement could contribute significantly to the defence of the mammary gland. A previous study established that the classical pathway of complement activation does not function in milk, as a result of the lack of C1q, unless inflammation develops

* For correspondence; e-mail: rainard@tours.inra.fr

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(Rainard & Poutrel, 1995). It has been hypothesized that the deposition of C3 on bacteria effected by normal milk resulted from the activation of the alternative pathway, because C4 was not deposited on bacteria, but direct evidence of the functioning of the alternative pathway was lacking. Furthermore, indirect evidence in favour of the intervention of the alternative pathway, like the requirements for $Mg^{2+}$ but not $Ca^{2+}$ ions, or the sensitivity to mild heat-treatment, did not yield conclusive results: selective chelation of $Ca^{2+}$ was shown to block both classical and alternative pathways in the C3 deposition test (Rainard & Boulard, 1992), and mild heat-treatment of milk (at 56 °C for 3–5 min or 50 °C for 45 min) did not suppress C3 deposition by milk.

The present study aimed to define the contribution of the alternative pathway to the deposition of C3 on bacteria in normal milk. To that end, antibodies were raised against a major protein of the alternative pathway (AP), complement factor B (fB), which is also the most heat-labile component of the complement system (Pang & Aston, 1978). These antibodies were used to specifically block the AP, and also to determine the concentration of fB in normal milk.

## MATERIALS AND METHODS

### *Animals and samples*

The study used 20 Holstein cows from the dairy herd of the Laboratory (Pathologie Infectieuse et Immunologie, Nouzilly). Cows in early and mid-lactation (between 1 and 5 months after calving) were selected on the basis of results of bacteriological examination of aseptically taken foremilk samples. Eligible cows had their four quarters free from infection by a major pathogen, and no more than one quarter infected with a minor pathogen (coagulase-negative staphylococci) without clinical signs. Only milk from uninfected quarters was used in the study. Aseptic milk samples were stored at 4 °C, centrifuged (1500 ***g***, 4 °C, 10 min) to remove cells and most of the cream, and skimmed milk was stored frozen in portions at −70 °C.

Blood was collected in evacuated tubes (Vacutainer®, Becton Dickinson, Rutherford, New Jersey, USA) from the tail veins of cows. To prepare serum, blood was allowed to clot at 37 °C for 1 h, then at 20 °C for another 2 h before centrifugation (1500 ***g***, 5 min). Pooled normal bovine serum (NBS) was prepared by mixing the sera of 12 of the 20 sampled cows.

### *Analysis of milk samples*

Somatic cell counts (SCC) in milk samples were determined electronically using a Fossomatic cell counter (Foss Food Technology, Hillerød, Denmark), according to standard procedures. Absence of bacteria was checked by spreading 25-$\mu$l portions of the milk sample on esculin blood agar plates. Usually encountered mastitis pathogens such as streptococci, staphylococci, coliforms and *Corynebacterium bovis* were detected after incubations of 24 and 48 h at 37 °C. Bovine serum albumin content in milk was determined by radial immunodiffusion as described by Poutrel *et al.* (1983).

### *Purification of complement fB*

In the absence of antiserum to bovine fB, the presence of fB in chromatographic fractions was assayed by the ability of fractions to restore the capacity of heated serum to deposit C3 fragments on complement-activating *Streptococcus agalactiae*. Precolostral calf serum (PCS) was heated at 56 °C for 3 min to destroy fB. PCS, which

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is virtually devoid of antibodies to *Str. agalactiae*, deposits C3 only by the alternative pathway, so that mild heating abrogates C3 deposition (Rainard & Boulard, 1992). To heated 10% PCS, 10 $\mu$l undiluted chromatographic fraction was added, and C3 deposition was assessed by ELISA (see below).

Bovine serum was fractionated with 20% (w/v) sodium sulphate. The supernatant was dialysed against 0·2 M-Na phosphate buffer, pH 6·0, containing 2 mM EDTA. The dialysed supernatant (75 ml) was loaded onto a cation exchange chromatography column (MonoS® HR 10/10, Pharmacia LKB Biotechnology AB, Uppsala, Sweden) equilibrated with the same buffer. Elution of bound proteins was achieved with a gradient of NaCl (0–0·3 M over 120 ml) in equilibrating buffer, applied with a system for fast protein liquid chromatography (BioLogic Chromatography System, BioRad Laboratories Inc., Hercules, California, USA). Functional fB was located in the third eluted peak (absorbance at 280 nm). Active fractions were pooled, diluted with 3 volumes of deionized water, brought to pH 7·2 and loaded onto an anion exchange column (MonoQ® HR 10/10, Pharmacia LKB) equilibrated in 0·02 M phosphate pH 7·2. Proteins were eluted with a gradient of NaCl (0–0·3 M over 60 ml). A major peak of absorbance at 280 nm was obtained, which coincided with fB functional activity.

The purity of fB was assessed by SDS-polyacrylamide gel (8%, w/v) electrophoresis using polyacrylamide under reducing conditions (Laemmli, 1970). Protein bands were visualized by staining with Coomassie Blue G-250 (Neuhoff *et al.* 1988). Before chromatography on the MonoQ column, three bands were visible, the major one at about 100 kDa. Analysis of the major peak eluted from the MonoQ column revealed only one band (Fig. 1), displaying the characteristic fB molecular weight of 100 kDa (Pang & Aston, 1978) and fB activity. The concentration of the purified fB was assessed with the NanoOrange® protein quantification kit (Molecular Probes, Inc, Eugene, Oregon, USA), using bovine serum albumin as a standard.

*Preparation of specific antibodies*

Antibodies were generated in two laying hens by three intramuscular injections of 100 $\mu$g of purified fB at 1-month intervals, the first one emulsified in complete Freund adjuvant (Difco laboratories, Detroit, Michigan, USA), the two booster injections in incomplete Freund adjuvant. IgY from egg yolk was purified as described by Akita & Nakai (1993), with some modifications. Briefly, egg yolks were pooled, and mixed with 9 volumes of water. The mixture was brought to pH 5·0 with 0·1 M-HCl and left overnight at 4 °C. After centrifugation (10000 ***g*** for 20 min at 4 °C), the supernatant was precipitated with 12% (w/v) polyethylene glycol and centrifuged (14000 ***g*** for 10 min). The pellet was resuspended in 0·01 M phosphate buffer pH 7·5, and precipitated with 25% ethanol at −20 °C. After a final centrifugation, the pelleted IgY were resuspended in phosphate buffer, filtered on 0·45 $\mu$m filter (Minisart, Sartorius AG, Göttingen, Germany) and stored frozen at −20 °C.

Antibodies were also obtained from rabbits after two intradermal injections (with a 3-week interval), at multiple sites, of purified fB (20 $\mu$g) emulsified in Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for the booster injection. An IgG fraction was prepared from the antiserum by affinity chromatography to staphylococcal protein A on an Econo-Pac® protein A cartridge (Bio-Rad), according to the manufacturer's recommendations. Part of the IgG fraction was biotinylated with sulpho-NHS-biotin (Pierce, Rockford, Illinois, USA), according to the manufacturer's instructions.

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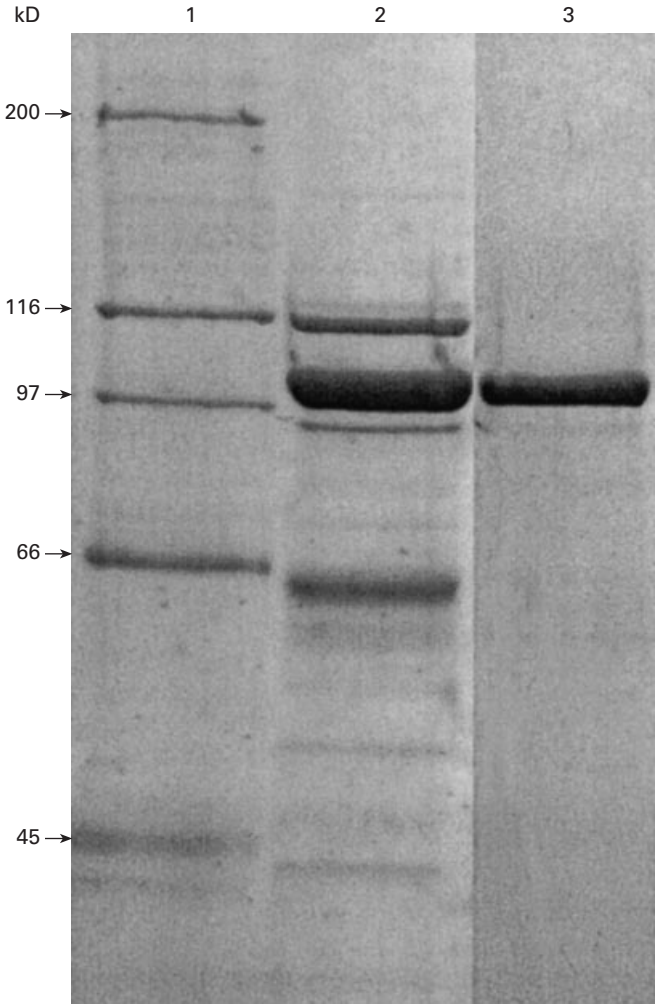



Fig. 1. SDS-polyacrylamide gel (8%) electrophoresis under reducing conditions of fB at different purification steps. Lane 1: marker proteins; lane 2: fraction containing fB after cation-exchange chromatography on MonoS column; lane 3: purified fB after anion-exchange chromatography on MonoQ column.

The specificity of antibodies was checked by double immunodiffusion in agar gel and indirect ELISAs, using either purified bovine IgG and C1q (Rainard & Poutrel, 1995), C3 and C5 (Rainard & Poutrel, 2000), and fB as antigens. Rabbit antibodies proved to be specific, but the IgY preparation cross-reacted with several bovine proteins, probably because of the presence of natural antibodies to bovine antigens, since IgY from unimmunized hens reacted in the same way. The IgY preparation was adsorbed by passage over an immunoadsorbent column made by coupling bovine serum depleted of fB by ion-exchange chromatography (as described above) to affinity gel (EAH-Sepahrose, Pharmacia-LKB Biotechnology) with 0·25% glutaraldehyde. After adsorption, IgY antibodies gave only one band without a spur in double immunodiffusion against whole bovine serum (Fig. 2) and reacted only with fB in the indirect ELISAs.

Complement component C1q was purified as described by Rainard & Poutrel

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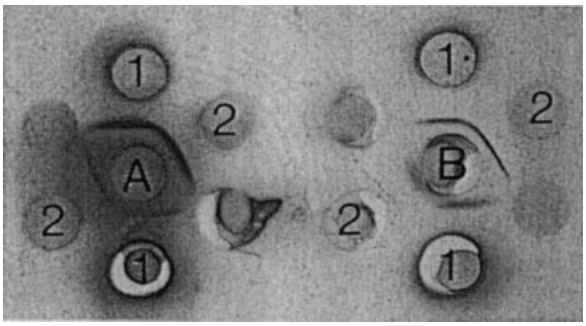



Fig. 2. Agar gel immunodiffusion analysis of the specificity of rabbit antiserum (A) and of hen IgY (B) to bovine fB. 1: pool of bovine serum (NBS). 2: purified fB.

(1995), and IgY anti-C1q prepared in the same way as IgY anti-fB (except for the last adsorption step).

*ELISAs to assess concentrations of complement components fB and C3*

ELISAs were performed in microtiter plates Nunc™ Immunoplate Maxisorp. Following each incubation step, the plates were washed 5 times with PBS supplemented with 0·1% (vol/vol) Tween 20 (Sigma Chemical Co., St Louis, Missouri, USA). Dilutions of sera and immune reagents were done in PBS containing 0·1% gelatin, 10 mM EDTA and phenol red (20 $\mu$g/ml) (PBSG). Appropriate dilutions of the immune reagents were dispensed in 100-$\mu$l samples to duplicate wells and incubated at 37 °C unless otherwise stated.

A sandwich ELISA was used to assess the concentrations of fB. The sequence of incubation steps was as follows: (1) coating of the plate with rabbit anti-bovine fB (5 $\mu$g/ml) diluted in 0·1 M carbonate buffer pH 9·6 overnight at 4 °C, (2) blocking with 0·5% gelatin for 30 min, (3) incubation with samples under test (normal milk was diluted 1/100 and serum at 1/5000) or a series of twofold dilutions (from 240 to 3·74 ng/ml) of purified fB for 1 h, (4) incubation with biotinylated IgG to fB diluted 1/2000 in PBSG for 30 min, (5) incubation with peroxidase-conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA) diluted 1/10000 for 30 min, and (6) incubation with a 2% dilution of 2,2′-azino-bis(3-ethylbenzthiazoline-sulphonate) (Sigma) in 0·1 M citrate buffer, pH 4·2, with the addition of 0·075% hydrogen peroxide just before use. Optical density (OD) was read at 414 nm. Wells that received PBS-gelatin instead of sample served as blanks. OD values given by the tested samples were referred to the standard curve obtained with the dilutions of purified fB.

Concentrations of C3 in milk were determined by ELISA (Rainard & Poutrel, 2000). Milk content was expressed as the percentage of values given in pooled normal bovine serum (NBS), made by mixing equal parts of the serum of the cows on experiment. Appropriate dilutions of NBS were used in the ELISA to establish the standard curve.

*ELISAs to assess deposition of complement components C3 and C4 on bacteria*

Deposition of C3 was assessed with whole bacterial cells immobilized in microtiter plates as described by Rainard & Boulard (1992). One strain of *Str. agalactiae* (443·31) was used as the assay microorganism for complement component deposition because

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it behaves like a particulate activator of the alternative pathway, and of the classical pathway in the presence of antibodies (Rainard & Boulard, 1992). Briefly, plates (Nunc™ Immunoplate Polysorp) were coated with a suspension of bacteria (OD 0·1 at 600 nm) and blocked with 0·5% gelatin. The sequence of incubations steps (at 37 °C), separated by five washes with PBS, was as follows: (1) either NBS diluted in RPMI 1640 buffered with 10 mM HEPES (N-2-hydroxyethlylpiperazine-N′-2-ethanesulphonic acid) and supplemented with 0·1% gelatin or undiluted milk for 2·5 h, (2) mouse monoclonal antibody (MD3) to bovine C3 for 30 min, (3) peroxidase-conjugated goat antibodies to mouse immunoglobulin G (Jackson Immunoresearch Laboratories) for 30 min, and (4) peroxidase substrate. Wells that received RPMI-gelatin instead of milk or serum served as blanks. A series of dilutions of NBS was incorporated in each plate and served to establish a reference curve. Results were expressed as percentage of C3 deposition relative to C3 deposition given by NBS.

Deposition of bovine C4 was evaluated as for the deposition of C3, except that rabbit antiserum to human complement C4 (cross-reacting with bovine C4) was used in step (2).

Dilution of serum or milk before assessment of deposition of complement components on bacteria was done in saline (0·15 M-NaCl) buffered with 10 mM HEPES and supplemented with 0·5% gelatin, 5 mM-$CaCl_2$ and 2 mM-$MgCl_2$ ($HSG^{2+}$).

*Statistical analysis*

Experimental data were analysed with the Spearman's rank correlation ($r_s$), a non-parametric test, using the software StatView (Abacus Concepts, Berkeley, California, USA).

## RESULTS

*Validation of the ELISA for fB*

Intra-assay coefficient of variation, calculated from 11 determinations of a milk sample, was 6·8%. The inter-assay coefficient of variation, which was determined by measuring milk samples on seven different plates, was 11·6%. OD values at 414 nm were related to fB concentrations (smoothed cubic spline fitting) between 1·5 and 160 ng/ml. Recovery of fB in milk was tested by adding 5 $\mu$g of purified fB to milk. ELISA yielded concentrations of fB of $2{\cdot}12 \pm 0{\cdot}23$ (SD) and $6{\cdot}81 \pm 0{\cdot}36$ $\mu$g/ml before and after addition, respectively ($P > 0{\cdot}1$). Interference of milk with the measurement of fB by ELISA was also tested by establishing the dilution curves of fB in milk, serum and buffer.

Dose-response curves of fB in serum and milk were parallel to the standard curve of purified fB in buffer (Fig. 3). This suggests that the ELISA assessed the same antigen in milk and serum, and indicates that milk did not interfere with the assay.

*Concentration of fB in normal milk*

Mean concentration of fB, determined in the milk of 42 uninfected quarters of 20 cows, was 2·06 $\mu$g/ml (Table 1) and values ranged from 0·96 $\mu$g/ml to 5·0 $\mu$g/ml. This represented 0·57% of the mean concentration of fB in the blood of these cows (360 $\mu$g/ml; range: 288–480 $\mu$g/ml), a percentage comparable to the percentage of BSA in milk (0·63%; 0·27 mg/ml *vs.* 43·2 mg/ml in serum) but much less than the 2·71% of C3 (Table 1). Correlations between the concentrations of fB and the concentrations of BSA or C3 in milk, although statistically significant ($P < 0{\cdot}01$), were low (Table 1).

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Table 1. *Mean concentrations of fB, C3, BSA and somatic cells in milk samples from 42 uninfected healthy quarters of 20 cows*

| | fB ($\mu$g/ml) | C3 (% of serum) | BSA (mg/ml) | Cells ($10^{-3}$/ml) |
|---|---|---|---|---|
| Mean | 2·06 | 2·71 | 0·27 | 38 |
| SEM | 0·14 | 0·13 | 0·02 | 12·7 |
| Correlation ($r_s$)† | | 0·464 | 0·546 | 0·392 |

$r_s$, Spearman's rank correlation between fB concentration and concentrations of the other measured parameters. All correlations were statistically significant ($P < 0{\cdot}01$). C3 concentrations are expressed as percent of the value found in pooled bovine serum.

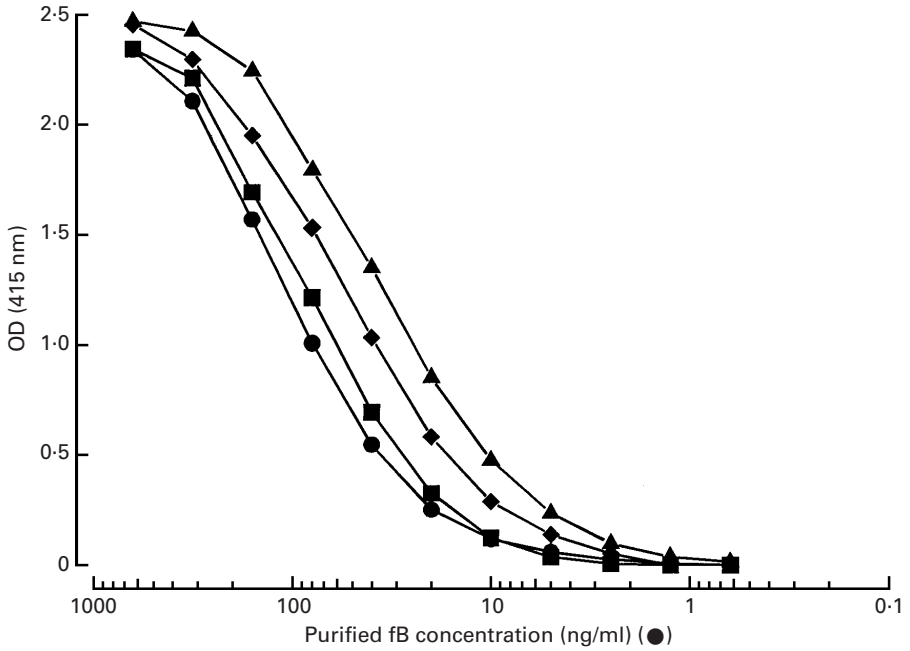



Fig. 3. Comparison of ELISA dilution curves of fB. Curves were obtained by diluting milk (■) or milk supplemented with 5 $\mu$g/ml purified fB (▲) (series of twofold dilutions from 1/5 to 1/5120), or NBS (◆) (twofold dilution starting at 1/2000) with PBSG. In parallel, purified fB was diluted (twofold dilutions from 640 to 0·625 ng/ml) in PBSG (●) to calibrate the ELISA. Concentrations of the Y axis apply only to the calibration curve. Dilutions of milk and serum were chosen to make the slope of the curves easier to compare.

*Contribution of fB to activation of complement in milk*

None of the milk samples allowed the deposition of C4 on bacteria (results not shown), which demonstrated that the classical pathway of complement activation was not operating.

The effect of IgY anti-fB on the capacity of milk to deposit C3 fragments on bacteria was tested on 15 quarter milk samples (Fig. 4). Increasing amounts of IgY caused a decreasing deposition of C3, with near complete inhibition when 10 $\mu$l IgY were added per well. At this dose, C3 deposition was restored with purified fB (4 $\mu$g/ml). IgY prepared in the same way, but from hens immunized with bovine C1q, did not inhibit C3 deposition (results not shown). Also, IgY anti-fB (10 $\mu$l in 100 $\mu$l

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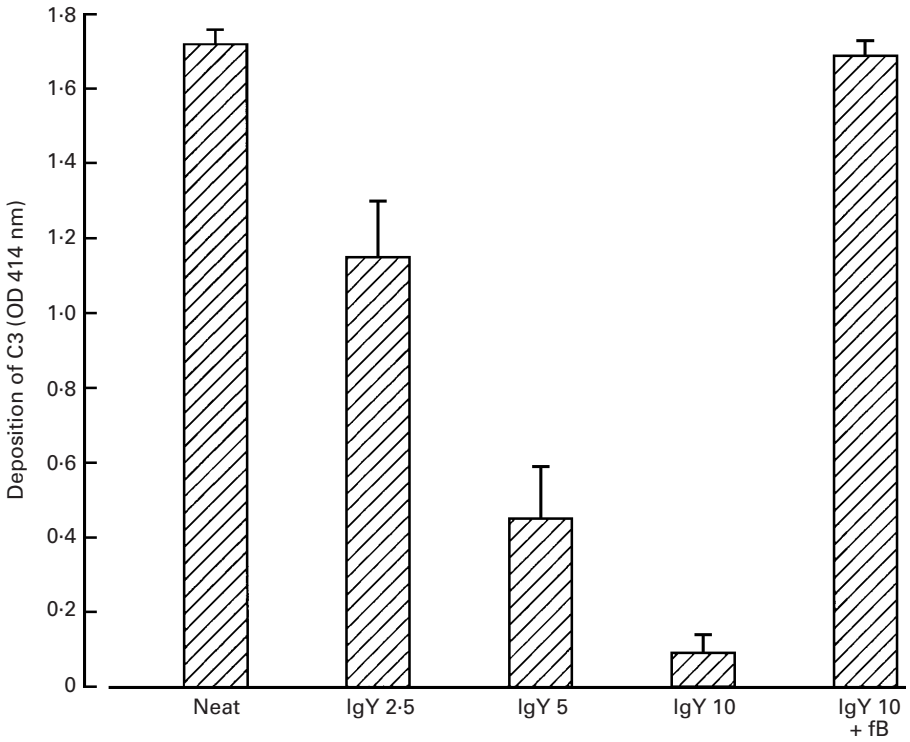



Fig. 4. Inhibition of deposition of C3 by IgY anti-fB. Deposition of C3 on *Str. agalactiae* was measured by ELISA. Bacteria were first incubated with neat milk (100 $\mu$l) or with milk supplemented with increasing amounts of IgY (2·5, 5 or 10 $\mu$l) or both IgY (10 $\mu$l) and fB (4 $\mu$g/ml, final concentration) for 30 min on melting ice. Then C3 deposition was allowed to take place for 2·5 h at 37 °C.

of serum dilution) did not inhibit the deposition of C3 and C4 exerted by 5% NBS (not shown), indicating that the IgY did not interfere with the classical pathway of activation.

The effect of addition of purified fB to milk on the deposition of C3 on bacteria was investigated. Preliminary experiments with different concentrations of fB added to pooled normal milk showed that a slight enhancement of deposition of C3 (OD augmented from 1·25 to 1·39) occurred at 5–10 $\mu$g/ml but that inhibition occurred at 25 $\mu$g/ml. Attempts were made with addition of fB (5 $\mu$g/ml) to milk either undiluted or diluted 1/2 or 1/4 in $HSG^{2+}$: OD values with added fB *vs.* no addition were 1·13 *vs.* 1·12, 1·02 *vs.* 1·05, and 0·56 *vs.* 0·49, respectively. Overall, these results indicated that addition of purified fB did not improve the deposition of C3 effected by normal milk.

*Effect of heat treatment on fB*

Heat treatment of serum is currently used to alter complement activity. Heating at 56 °C for 30–45 min is used to abrogate complement activity, whereas heating at 56 °C for 3–5 min or at 50 °C for 45 min is used to selectively interrupt the alternative pathway (Pang & Aston, 1978). The effect of heat treatment on fB and the alternative pathway in milk was tested by measurement of C3 deposition and fB antigenicity (ELISA reactivity), and compared with the effect of the same treatments

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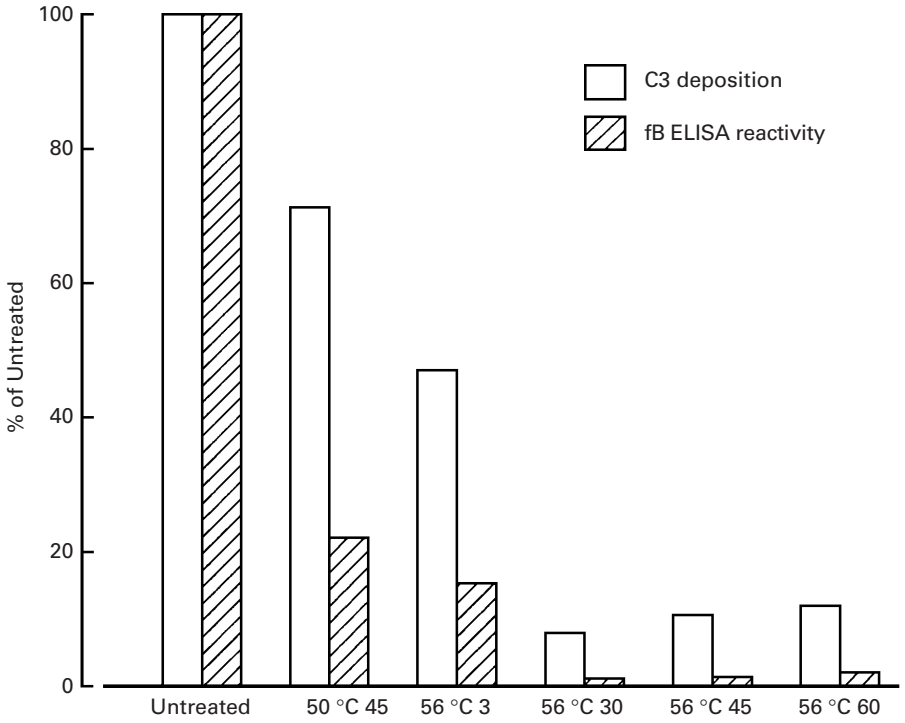



Fig. 5. Effect of heat-treatment on the C3 deposition effected by NBS and the apparent concentration of fB (fB reactivity), measured with their respective ELISA. NBS was heated in a water bath at 50 °C for 45 min or at 56 °C for 3, 30, 45, or 60 min, and tested by ELISA (diluted 20-fold for C3 deposition, and 2500-fold for the fB ELISA). Results are expressed as percent of the values given by untreated NBS.

in PCS and NBS. In PCS, all the treatments completely inhibited C3 deposition (not shown) as expected since only the alternative pathway operates in the absence of antibodies to *Str. agalactiae* (Rainard & Boulard, 1992). In contrast, mild heat treatment only partially inhibited C3 deposition exerted by NBS, whereas heating at 56 °C for 30 min or more abrogated C3 deposition (Fig. 5). ELISA reactivity of fB was completely lost in PCS with all of the treatments, suggesting that loss of antigenicity paralleled loss of functional activity. In milk, the behaviour of fB was quite different (Fig. 6). The two milder treatments left most of C3 deposition capacity intact, and 45 min or 1 h of heating at 56 °C were necessary for complete inhibition. Even more striking was the preservation of antigenicity of fB, which remained intact after the two milder treatments and was only halved by the harsher treatments.

Heat stability of purified fB in milk was checked by supplementing milk with purified fB before heat-treatment. ELISA was used to compare the recoveries of fB in milk, buffer (PBSG) and serum (NBS) (Table 2). As expected, some fB was recovered in NBS after heating at 50 °C for 45 min, but fB was destroyed by heating at 56 °C for 30 or 45 min. In PBSG, fB resisted heating at 50 °C for 45 min, but most of the ELISA activity disappeared by heating at 56 °C for 30 min or more. In contrast, recovery of fB exceeded 60 % in milk, and there was no difference between fB intrinsic to milk or purified fB added to milk.

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Table 2. *Percentage recovery of fB by ELISA in milk, serum and buffer as a function of heat-treatment*

| | Milk | | Milk + fB 5 μg/ml | | NBS | | PBSG | |
|---|---|---|---|---|---|---|---|---|
| Heat-treatment | [fB]† | Recovery % | [fB] | Recovery % | [fB] | Recovery % | [fB] | Recovery % |
| None | 2·39 | 100 | 7·55 | 100 | 796 | 100 | 5·25 | 100 |
| 50 °C, 45 min | 2·44 | 102 | 7·75 | 103 | 304 | 38 | 5·80 | 110 |
| 56 °C, 30 min | 1·75 | 73 | 4·75 | 63 | 9·71 | 1 | 0·79 | 15 |
| 56 °C, 45 min | 1·52 | 64 | 4·61 | 61 | 16·32 | 2 | 0·62 | 12 |

† Concentration of fB (μg/ml).

Purified fB was added to milk or buffer (PBS) and submitted to heat-treatment, along with the milk and pooled NBS. Recovery of fB was measured by ELISA to assess the stability of fB as a function of its environment.

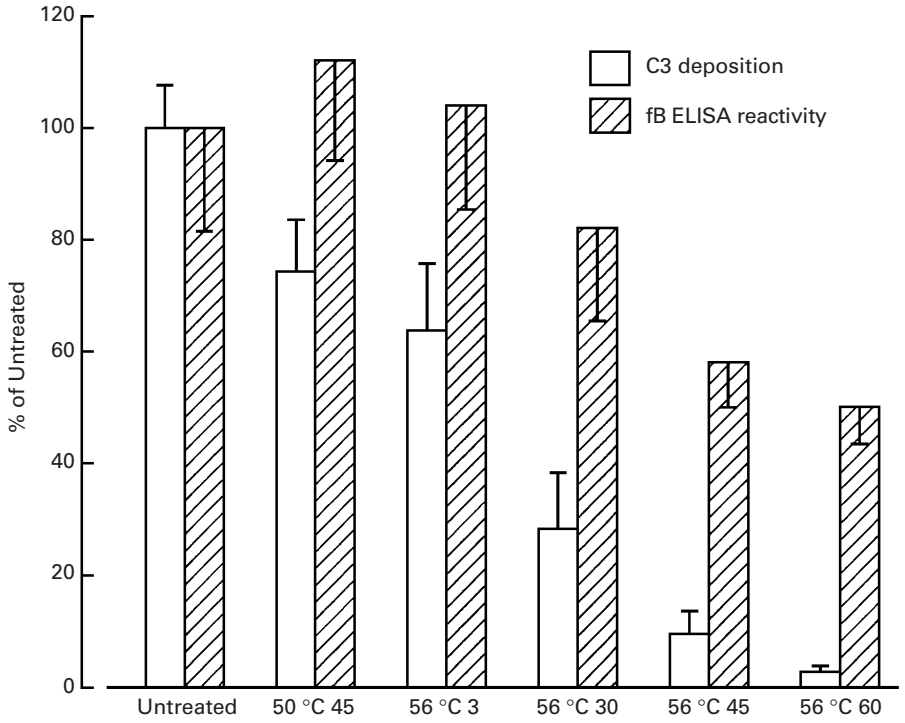



Fig. 6. Effect of heat-treatment of six milk samples on C3 deposition and the apparent concentration of fB (fB reactivity), measured with their respective ELISA. Milks were heated as indicated above, and tested by ELISA (undiluted for C3 deposition, diluted 50-fold for fB reactivity). Results are expressed as percent of the values given by untreated milk (mean ± SEM).

## DISCUSSION

Purification of bovine fB and the development of a sandwich ELISA enabled the measurement of the concentrations of this complement component in blood and milk. The previously reported concentration of fB in bovine blood (Tabel *et al.* 1983, 1984) is comparable (342 μg/ml) to the concentration found in the present study (360 μg/ml). In both cases the animals were Holstein cows. In human serum, the normal concentrations extend from 74 to 286 μg/ml (Oglesby *et al.* 1988). The author is not aware that the presence of fB in milk has been reported previously. The concentration found in normal milk (2·06 μg/ml) represents 0·57 % of the serum

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concentration, which is comparable to the proportion of BSA (0·63 %) but lower than the proportion of C3 (2·7 %). This suggests that fB might permeate from blood to milk, as BSA is assumed to do. Nevertheless, the correlation between fB and BSA concentrations in milk was rather low ($r_s = 0{\cdot}546$), so that no conclusion can be drawn as to the origin of milk fB. Complement fB is synthesized by a variety of cells including intestinal epithelial cells, monocytes and macrophages, and its synthesis is regulated by cytokines and growth factors (Hogasen *et al.* 1995; Andoh *et al.* 1999). Synthesis of fB by mammary epithelial cells cannot be excluded and deserves investigation.

In contrast to mammalian antibodies, chicken antibodies of the IgY class do not activate the complement system of mammals (Larsson *et al.* 1993). It is thus possible to use IgY directed against a complement component of the bovine system, to block the corresponding activation cascade without causing consumption of complement by initiating the classical pathway of activation. Consequently, the inhibition of C3 deposition by the IgY anti-fB can be interpreted as an evidence that fB was involved in the process. The restoration of C3 deposition with purified fB and the absence of effect of IgY anti-C1q support the specificity of the inhibition. The classical pathway was not operational in milk in the absence of inflammation (Rainard & Poutrel, 1995), but the indirect means used in that experiment to suggest the functioning of the alternative pathway yielded inconclusive results: after desalting, C3 deposition could not be restored with $Mg^{2+}$, or $Ca^{2+}$ and $Mg^{2+}$ ions, activity resisted heat treatment of the milk, and preferential chelation of $Ca^{2+}$ inhibited C3 deposition on bacteria despite the presence of $Mg^{2+}$ ions. The present results show that the alternative pathway was the only complement pathway operating in normal milk.

Bovine milk contains sizeable amounts of C3, about 2·5 % of the concentration in serum (Mueller *et al.* 1982; Rainard & Poutrel, 2000), comparable to the 2·7 % found in the present study. The relatively low milk concentration of fB compared with C3 suggested that fB could be a limiting factor for the alternative pathway in milk. Attempts to enhance the deposition of C3 on activating bacteria by adding purified fB were unsuccessful, indicating that fB was not a limiting factor. It has been shown that the alternative pathway of complement activation is highly sensitive to the equilibrium between individual components (fB, C3, D) and control proteins (H and I) (Nydegger *et al.* 1978). The present results suggest that fB was in sufficient amount in normal milk to insure the balance between the complement components of the alternative pathway.

This study showed that the resistance of fB to heat was dramatically increased in milk (Fig. 3). That fB behaved differently when heat-treated, as a function of its environment, was shown previously (Pang & Aston, 1978): purified fB was not inactivated after heating at 50 °C for 90 min, whereas in all bovine sera fB was inactivated by heating at this temperature for 45 min. Recoveries of fB with the ELISA agreed with these observations, showing a greater heat-susceptibility of fB in serum than in buffer. They also confirmed the resistance to heating at 56 °C (> 60 % recovery; Table 2) of fB purified from serum when diluted in milk. The protection afforded to fB by milk can explain the resistance of C3 deposition activity of milk to heating at 56 °C for 3 min or at 50 °C for 45 min, although the classical pathway was not functioning.

The discrepancy between complete inhibition of C3 deposition by heating of milk at 56 °C for 45 min or more, but preservation of about 50 % of fB antigenicity (Fig. 6), may have two explanations. Loss of functional activity may precede loss of antigenicity, i.e. a partial denaturation may allow recognition by some of the

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antibodies of the antiserum while fB is no longer functional. It may also suggest that, in the case that functionality of fB parallels its antigenicity, another complement component of the alternative pathway was denatured by heat before fB.

In conclusion, fB appeared necessary for deposition of C3 in normal milk. Although present in milk in relatively low concentration, fB was apparently not a limiting factor for the functioning of the alternative pathway. Protection of fB by milk is of practical interest: because of the increased heat-resistance of fB, mild heating of milk to selectively inhibit the alternative pathway of complement activation is an invalid method. On the contrary, it is necessary to heat milk for at least 45 min to completely inactivate complement. The knowledge of the relative heat resistance of complement in milk will be useful to assess the contribution of milk complement to opsonization of mastitis-causing bacteria.

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