

## Bovine milk fat globules do not inhibit C5a chemotactic activity

Pascal RAINARD\*

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

(Received 12 December 2001; accepted 4 March 2002)

**Abstract** – The C5a complement fragment is a potent inflammatory molecule but its contribution to the inflammatory response of the mammary gland remains uncertain. One of the unresolved questions is the possible interference of whole milk with C5a. In this study, the chemotactic activity of purified bovine C5a was tested in the presence of whole or skimmed milk. Milk from healthy glands acted as a chemoattractant, which could mask any inhibitory effect on C5a activity. To circumvent milk activity, washed milk fat globules were incubated with optimal (1 nM) or suboptimal (0.1 nM) concentrations of C5a, and the eventual chemoattractant activity was measured. There was no reduction in C5a-induced migration through a polycarbonate filter or shape-change of neutrophils. The concentrations of C5a determined by ELISA in the fluid phase were not reduced after incubation with the fat globules. It can be concluded that bovine milk fat globules do not trap or degrade C5a. Although these results do not explain the inhibitory effect of whole milk in the C5a-induced recruitment of neutrophils in milk, they suggest that milk does not inhibit the second major activity of C5a (apart from chemotaxis), i.e. the stimulation of phagocytic and bactericidal activities of neutrophils.

### C5a / chemotactism / milk / fat globule

**Résumé** – Les globules gras du lait de vache n'inhibent pas l'activité chimiotactique du C5a. Le fragment du complément C5a est un puissant agent inflammatoire mais sa contribution à la réponse inflammatoire de la glande mammaire reste incertaine. Une des questions non résolues est l'interférence possible du lait entier avec le C5a. Dans cette étude, l'activité chimiotactique du C5a bovin purifié a été évaluée en présence de lait entier ou écrémé. Le lait de glandes saines s'est révélé être un chimioattractant, ce qui pourrait masquer un éventuel effet inhibiteur sur le C5a. En vue de contourner l'activité du lait, des globules gras lavés ont été incubés avec des concentrations optimales (1 nM) ou suboptimales (0,1 nM) de C5a, et l'activité résultante a été mesurée. La migration à travers une membrane filtrante de polycarbonate ou la déformation des polynucléaires neutrophiles n'ont pas été diminuées. Les concentrations de C5a, mesurées par ELISA, n'ont pas été réduites après incubation

---

\* Correspondence and reprints  
Tel.: (33) 2 47 42 76 33; fax: (33) 2 47 42 77 79; e-mail: rainard@tours.inra.fr

avec les globules gras. Il peut en être conclu que les globules gras du lait ne capturent pas ou ne dégradent pas le C5a. Bien que ces résultats n'offrent pas d'explication à l'effet inhibiteur du lait entier sur le recrutement C5a-dépendant des neutrophiles dans le lait, ils suggèrent que le lait n'inhiberait pas l'autre activité majeure du C5a (en plus du chimiotactisme), à savoir la stimulation des fonctions phagocytaires et bactéricides des neutrophiles.

## **C5a / chimiotactisme / lait / globules gras**

### **1. INTRODUCTION**

Activation of the complement system generates several inflammatory fragments, among which C5a is the most active. This molecule is chemotactic for granulocytes and monocytes *in vitro* and is a potent mediator of inflammation *in vivo* [6]. The contribution of C5a to the recruitment of phagocytes in the milk of infected mammary glands has been addressed by several studies. Concentrations of C5a (around 50 ng/mL) can be found in the milk of quarters suffering from clinical mastitis [17]. This concentration of C5a has been shown to induce the migration of bovine PMN through a confluent monolayer of mammary epithelial cells [18]. Moreover, infusion of 65 ng of C5a into the teat cistern of cows provokes an influx of PMN [13]. Other studies have shown that zymozan activated plasma (ZAP), a source of C5a, elicits cell recruitment when infused into the mammary glands of non-lactating ewes or cows [3]. Nevertheless, the same amount of ZAP remained without an effect when infused into lactating glands [4]. The authors showed that by mixing ZAP with whole ewe milk, the *in vivo* inflammatory activity is inhibited, whereas mixing it with skimmed milk is without an inhibitory effect. This observation suggests that a component of whole milk, but not skimmed milk, interferes with the inflammatory activity of C5a. Fat globules are the likely candidates for such inhibition. This is compatible with the activity of purified C5a in the teat cistern of cows, since in this model, based on the surgical separation of the teat cistern from the udder

cistern, the tested inflammatory agents are not in contact with milk [13].

The present study was aimed at testing the hypothesis of the interference of milk fat globules with C5a. The surface of fat globules is partly covered with remnants of epithelial cell membranes, and consequently may harbour receptors present at the apical surface of the secretory cells. It has been shown that epithelial cells of the lung possess receptors for C5a [10]. Providing that mammary epithelial cells express receptors for C5a, these receptors could be present on fat globules, and could trap molecules of C5a in milk. Another possibility is that a protease/peptidase on fat globules could inactivate C5a. The effect of whole milk or separated fat globules on purified bovine C5a was investigated through the chemotactic activity of C5a for PMN and the capture of C5a by fat globules.

### **2. MATERIALS AND METHODS**

#### **2.1. Animals and milk samples**

Milk samples from 6 healthy Holstein cows were used for the experiments. These cows were selected on the basis of the absence of mammary infections (at least two consecutive bacteriologically negative quarter milk samples) and inflammation (less than  $10^5$  cells/mL in quarter milk samples). Cows were in early or mid-lactation (1 to 6 months) and were milked twice daily. Two quarters per cow were arbitrarily selected for the foremilk samples.

## 2.2. Isolation of PMN

Bovine PMN were isolated from the peripheral blood as described [2] after hypotonic lysis of erythrocytes and resuspended in RPMI 1640 without  $\text{NaHCO}_3$  (Biocrom KG, Berlin, Germany) supplemented with 0.1% bovine serum albumin (low endotoxin, low IgG quality; Sigma Chemical Co., St Louis, Mo, USA) and 20 mM HEPES (RPMI-AH) at a concentration of  $2 \times 10^6/\text{mL}$ . More than 95% of the cells excluded trypan blue and were spherical, as determined by light microscopy.

## 2.3. Preparation of skimmed milk and fat globules

In order to test the suspected interaction of milk fat globules with C5a, fat globules were separated from milk by centrifugation ( $1\,000 \times g$ , 30 min,  $4^\circ\text{C}$ ). The hardened fat layer was removed with a spatula and gently resuspended in 10 volumes of warm ( $37^\circ\text{C}$ ) RPMI-AH. The suspension of fat globules was chilled on melting ice and centrifuged ( $1000 \times g$ , 30 min,  $4^\circ\text{C}$ ). After two washings, the washing liquid was devoid of chemotactic activity (tested by the shape change assay). Purified C5a diluted in RPMI-AH (0.1 or 1 nM) was incubated for 1 h at  $37^\circ\text{C}$  with an amount of washed fat globules corresponding to an equivalent volume of milk. After centrifugation, the liquid phase was used in the migration test.

## 2.4. Chemotaxis assay

Locomotion of PMN was measured using a 48-well microchemotaxis chamber (Neuroprobe, Bethesda, MD, USA) in which a  $5\text{-}\mu\text{m}$  pore-size polycarbonate PVP-free filter (Poretics Corp., Livermore, USA) separates the upper and lower chamber, as described [7]. Briefly, the filter was prewetted in RPMI-AH just before assembly of the chamber. Lower wells were filled with  $28\ \mu\text{L}$  of the medium containing the

chemoattractant to be tested or the control medium (RPMI-AH). Purified bovine C5a was used at concentrations of 0.01, 0.1, 1 and 10 nM, and milk (whole or skimmed) was used without dilution. Assays were made in duplicate. Upper wells received  $50\ \mu\text{L}$  of the cell suspension ( $2 \times 10^6$  PMN/mL). Cells were allowed to migrate for 1 h at  $37^\circ\text{C}$ , then the filters were fixed in methanol and stained with May-Grünwald-Giemsa stain. PMN adhering to the lower surface of the filter were quantified by counting the cells in five random microscope fields (magnification  $400\times$ ). Cell migration was expressed as the mean number of adherent cells per microscope field.

## 2.5. Shape change assay of PMN

Elongation of PMN was evaluated with a method [9] adapted to 96-well microtiter plates [15]. Briefly, samples of  $90\ \mu\text{L}$  of the chemoattractant to be tested diluted in RPMI-AH were distributed in wells of gelatin-coated microtiter plates. Before incubation (15 min at  $37^\circ\text{C}$ ),  $10\ \mu\text{L}$  of PMN suspension ( $2 \times 10^6/\text{mL}$ ) were added. Plates were examined under an inverted microscope. In the presence of a chemoactive agent, the morphology of PMN changes from a spherical to an elongated, irregular shape. The percentage of elongated cells was recorded. Controls included wells with cells in RPMI-AH (negative) and wells with cells and 1 nM C5a (positive).

## 2.6. ELISA for C5a

The purification of bovine C5a and the quantification of C5a by ELISA have been described previously [15]. Purified C5a was essentially C5a<sup>desArg</sup> but is designated as C5a throughout the text to simplify.

**2.7. Statistical analyses**

The unpaired Student *t*-test was used to determine the statistical significance of the differences between the results. *P* values greater than 0.05 were considered not significant.

**3. RESULTS**

The maximum chemotactic activity of purified C5a was found at 1 nM and 0.1 nM C5a, whereas C5a at 0.01 nM was barely active (Tab. I). This result is in agreement with the previously reported activity of purified bovine C5a [8]. Dilution of 0.1 nM C5a in whole milk did not reduce the migration of PMN through the filter (Tab. I). Controls with the whole or skimmed milk in which C5a was omitted showed that milk induced migration of PMN. All of the 12 milk samples tested (from different quarters of 6 cows) induced migration.

To test the possible interaction of C5a with milk fat globules, purified bovine C5a in RPMI-AH was incubated with an amount of washed fat globules corresponding to an equivalent volume of milk. After incubation, the chemotactic activity of the liquid phase was analysed with the polycarbonate filter-based assay. There was no statistically significant reduction of migration of PMN (Tab. II).

The potential capacity of fat globules to interfere with the activity of C5a was also studied with the shape change assay. The activity of purified C5a was titrated in the assay and washed fat globules resuspended in a volume of RPMI-AH corresponding to half the volume of initial milk (twice concentrated fat) were incubated with C5a at the two lowest dilutions inducing cell polarisation. Percentages of cells undergoing shape change were not modified by pre-incubation with fat globules (Fig. 1). Concentrations of C5a before and after incubation with fat globules were determined by ELISA. With 1 nM C5a, concentrations ( $\pm$ SD) of  $8.26 \pm 0.22$  ng/mL were obtained before incubation. After incubation with fat globules from 5 cows, the mean concentration was  $8.57 \pm 0.71$  ng/mL. With 0.1 nM, concentrations of  $0.81 \pm 0.28$  and  $0.83 \pm 0.02$  ng/mL were found before and after incubation, respectively. These values are not significantly different.

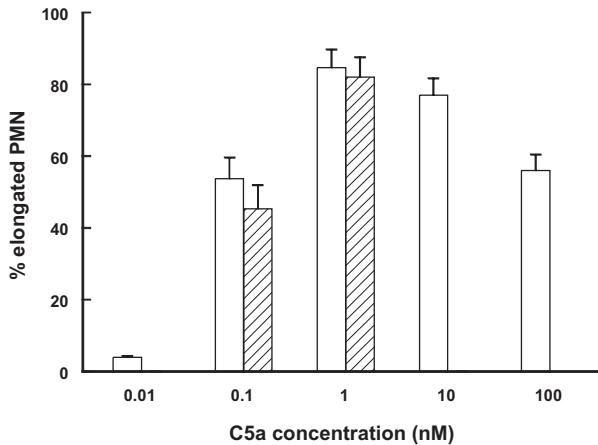
**4. DISCUSSION**

This investigation was undertaken in order to test the hypothesis of the inhibition of the activity of C5a by milk fat globules. It was prompted by the observation that whole milk, but not skimmed milk, inhibited the recruitment of cells induced by the infusion of ZAP, a source of C5a, into the mammary glands of non-lactating ewes [4].

**Table I.** Chemotactic activity of purified C5a or milk on PMN.

Chemoattractant		Chemotactic activity (No. of cells per field $\pm$ SD)
C5a	10 nM	16.2 $\pm$ 3.1
	1 nM	24.6 $\pm$ 2.8
	0.1 nM	19.9 $\pm$ 4.2
	0.01 nM	2.8 $\pm$ 2.4
	0.1 nM + whole milk	22.5 $\pm$ 5.7
Whole milk		14.2 $\pm$ 4.3
Skimmed milk		17.5 $\pm$ 4.6

Chemotactic activity was measured with the polycarbonate filter based assay.



**Figure 1.** Effect of milk fat globules on the chemotactic activity of C5a. The dose-effect of C5a on PMN shape-change (transition from a round to an elongated shape) was measured with the microtiter plate assay (empty bars). The effect of pre-incubating C5a with washed fat globules was measured at the optimal and sub-optimal active C5a concentrations (hatched bars). Results are means  $\pm$  SEM of 6 distinct experiments performed in duplicate.

**Table II.** Effect of pre-incubation of C5a with milk fat globules on the migration of PMN through a polycarbonate filter.

Pre-incubation with fat globules	C5a concentration	
	0.1 nM	1 nM
no	15.8 $\pm$ 4.0	25.3 $\pm$ 3.3
yes	14.1 $\pm$ 5.8	19.2 $\pm$ 5.7

Results are from 6 milk samples from 3 cows (2 quarters per cow). Values are the mean numbers ( $\pm$  SD) of cells per microscope field. The effect of pre-incubation with fat globules was not statistically significant.

The inflammatory complement fragment C5a has been shown to induce the influx of neutrophils in the lumen of the mammary glands of ruminants, provided they are not secreting milk, i.e. non-lactating [3, 4] or are prevented from contact with milk by a surgical procedure [13]. The refractory status of lactating glands could result from the fact that C5a cannot cross the epithelial barrier (cells and tight junctions) during lactation, preventing C5a to reach the responsive cells. This hypothesis supposes that mammary epithelial cells lack a receptor for C5a and are thus unresponsive to this inflammatory agent. After cessation of milking, the

epithelium lining is disorganised and, as a consequence, C5a could penetrate the mammary tissue and trigger an inflammatory response. This hypothesis could account for the differing responses of lactating and non-lactating mammary glands to C5a, but does not explain why whole milk inhibits the effect of C5a in non-lactating glands.

A simple explanation for the inhibition by whole milk is that fat globules interfere with C5a activity. It is known that proteases like plasminogen are associated with fat globule membranes [11]. Peptidases associated with fat globules could inactivate

C5a. Another possibility is that fat globule membranes, which originate from secretory epithelial cells, bear receptors for C5a, and may act as a sink for this molecule. Epithelial cells of the lung have been shown to possess receptors for C5a [10], but the presence of C5a receptors on the apical surface of mammary epithelial cells remains to be documented. In vitro experiments with purified bovine C5a have shown that C5a (> 25 ng/mL) provokes the migration of neutrophils through a confluent monolayer of mammary epithelial cells [18]. In vivo infusion of C5a in the teat cistern of cows also induces a moderate recruitment of neutrophils [13]. This could be interpreted as demonstrating the responsiveness of mammary epithelial cells to C5a, although the integrity and impermeability of the epithelium layer could not be guaranteed in those investigations. The results of the present study indicate that milk fat globules, at the concentrations present in milk, did not trap or degrade C5a in a detectable manner. Another component of whole milk which could trap or degrade C5a are milk cells. It is nevertheless dubious that the reported number of cells in milk (about  $10^5$  per mL), could have interfered with the high amounts of complement (0.5 mL of ZAP, which could contain up to 2 µg of C5a, for 4.5 mL of whole milk) used in the study demonstrating the inhibitory property of whole milk [4].

Another possibility is that the isolation and washing of milk fat globules eliminated an inhibitory component such as the anti-inflammatory low molecular weight component reported previously [12]. This component, if it exists, could have been adsorbed to the fat globules so that it would have been removed from milk during the skimming process, but would have been desorbed from the fat globules by the washings with RPMI-AH. The washings were necessary to get rid of the chemotactic activity of milk, which interfered consistently with the chemotaxis and shape-

change assays. Chemotactic activity of normal milk (as opposed to milk from inflamed glands) has been detected previously [5, 14]. It has been shown to be a low molecular weight molecule [1], but it is neither IL-8 [1] nor C5a [16].

At present, it seems difficult to piece together the experimental data relating to the inflammatory activity of C5a in the lumen of the mammary gland. Answers to specific questions such as the presence of receptors for C5a on the apical surface of mammary epithelial cells need to be obtained before a clear picture of the recruiting and activating activities of C5a in the mammary gland is available. The present study was a step in that direction, by showing that milk fat globules do not interfere with the activity of C5a.

## ACKNOWLEDGEMENTS

I am grateful to Henri Salmon for his help with the chemotaxis assay.

## REFERENCES

- [1] Barber M.R., Yang T.J., Chemotactic activities in nonmastitic and mastitic mammary secretions: presence of interleukin-8 in mastitic but not nonmastitic secretions, *Clin. Diagn. Lab. Immunol.* 5 (1998) 82-86.
- [2] Carlson G.P., Kaneko J.J., Isolation of leukocytes from bovine peripheral blood, *Proc. Soc. Exp. Biol. Med.* 142 (1973) 853-856.
- [3] Colditz I., Watson D., The role of humoral and cellular mediators in enhanced mammary inflammatory reactions to staphylococcal infection in systemically immunized ewes, *Microbiol. Immunol.* 26 (1982) 1171-1180.
- [4] Colditz I.G., Maas P.J.C.M., The inflammatory activity of activated complement in ovine and bovine mammary gland, *Immunol. Cell Biol.* 65 (1987) 433-436.
- [5] Craven N., Chemotactic factors for bovine neutrophils in relation to mastitis, *Comp. Immunol. Microbiol. Infect. Dis.* 1 (1986) 29-36.
- [6] Damereau B., Biological activities of complement-derived peptides, *Rev. Physiol. Biochem. Pharmacol.* 108 (1987) 151-206.

- [7] Falk W., Goodwin R.H., Leonard R.J., A 48-well microchemotaxis assembly for rapid accurate measurement of leukocyte migration, *J. Immunol. Methods* 33 (1980) 239-257.
- [8] Gennaro R., Simonic T., Negri A., Mottola C., Secchi C., Ronchi S., Romeo D., C5a fragment of bovine complement. Purification, bioassays, amino-acid sequences and other structural studies, *Eur. J. Biochem.* 155 (1986) 77-86.
- [9] Haston W.S., Shields J.M., Neutrophil leukocyte chemotaxis: a simplified assay for measuring polarising responses to chemotactic factors, *J. Immunol. Methods* 81 (1985) 229-237.
- [10] Haviland D.L., McCoy R.L., Whitehead W.T., Akama H., Momenti E.P., Brown A., Haviland J.C., Parks W.C., Perlmutter D.H., Wetsel R.A., Cellular expression of the C5a anaphylatoxin receptor (C5aR): demonstration of C5aR on nonmyeloid cells of the liver and lung, *J. Immunol.* 154 (1995) 1861-1869.
- [11] Hofmann C.J., Keenan T.W., Eigel W.N., Association of plasminogen with bovine fat globule membrane, *Int. J. Biochem.* 10 (1979) 909-917.
- [12] Ormrod D.J., Miller T.E., A low molecular weight component derived from the milk of hyperimmunized cows suppresses inflammation by inhibiting neutrophil emigration, *Agents Actions* 37 (1992) 70-79.
- [13] Persson K., Larsson I., Hallén Sandgren C., Effects of certain inflammatory mediators on bovine neutrophil migration in vivo and in vitro, *Vet. Immunol. Immunopathol.* 37 (1993) 99-112.
- [14] Rainard P., Effect of milk or colostrum on phagocytosis of glass- or plastic-adherent *Streptococcus agalactiae* by bovine granulocytes, *Vet. Immunol. Immunopathol.* 26 (1990) 203-208.
- [15] Rainard P., Sarradin P., Paape M.J., Poutrel B., Quantification of C5a/C5a<sup>desArg</sup> in bovine plasma, serum and milk, *Vet. Res.* 29 (1998) 73-88.
- [16] Riollet C., Rainard P., Poutrel B., Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*, *Clin. Diagn. Lab. Immunol.* 7 (2000) 161-167.
- [17] Shuster D.E., Kehrl M.E., Rainard P., Paape M.J., Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with *Escherichia coli*, *Infect. Immun.* 65 (1997) 3286-3292.
- [18] Smits E., Cifrian E., Guidry A.J., Rainard P., Burvenich C., Paape M.J., Cell culture system for studying bovine neutrophil diapedesis, *J. Dairy Sci.* 79 (1996) 1353-1360.