Lait 83 (2003) 101–110

DOI: 10.1051/lait:2003001

Original article

Proliferation and immunoglobulin production of hybridoma cells cultured on mastitic whey

Fatima MOUSSAOUI*, Yves LE ROUX, François LAURENT

Laboratoire de Sciences Animales, (Unité associée INRA), École Nationale Supérieure d'Agronomie et des Industries Alimentaires (ENSAIA),
2, avenue de la Forêt de Haye, BP 172, 54505 Vandoeuvre-Lès-Nancy, France

(Received 19 December 2001; accepted 16 September 2002)

Abstract – Lipopolysaccharide experimental mastitis was performed in order to obtain the same milk composition change involved in a clinical mastitis without, however, any bacterial proteolytic participation. Whey from milk with a high somatic cell count (SCC) had a lower mitogenic effect on anti-human prolactin-hybridoma (anti-hPL) cell culture but a greater immunostimulating effect than that of the fetal calf serum regularly used in cell culture. This immunostimulating effect was more pronounced when expressed per cell, with an effect two- to three- fold higher than that of fetal calf serum ($P < 0.01$), which illustrated the efficiency of such a complement.

Cell culture / whey / proteolysis / mastitis

Résumé – **Prolifération et production d'immunoglobulines d'hybridomes cultivés sur lactosérum de mammite.** Une mammite expérimentale à lipopolysaccharide a été menée en vue d'obtenir le même changement de composition du lait que celui observé dans les cas de mammite clinique, en éliminant toutefois toute interaction avec la protéolyse bactérienne. Le lactosérum issu d'un lait à fort dénombrement cellulaire (DC) avait un effet mitogénique plus faible sur une culture cellulaire d'hybridomes anti-prolactine humaine (anti-hPL) mais toutefois un effet immunostimulant plus important que le sérum fœtal de veau. Cet effet immunostimulant a été plus marqué lorsqu'il était exprimé par cellule, avec un effet 2 à 3 fois supérieur à celui observé avec le sérum de veau fœtal ($P < 0,01$), traduisant l'efficacité de la complémentation par un lactosérum issu d'un lait à fort DC.

Culture cellulaire / lactosérum / protéolyse / mammite

1. INTRODUCTION

Mastitis is an inflammation of the mammary gland that represents a great loss for milk producers, as well as in the dairy product industry. On this basis, an early detection device has been developed in order to prevent such losses; at the same time, valorization of milk unfit for consumption could be considered.

* Correspondence and reprints
Tel.: (33) 3 83 59 58 89; fax (33) 3 83 59 58 04; e-mail: toulouse31@hotmail.com

On a more fundamental basis, mastitis involves three phenomena: a decrease of synthesis [22, 24], an increase in permeability of the blood-milk barrier [24, 28] and an increase in proteolytic activity in milk [16, 24]. In the case of a lipopolysaccharide (LPS) infusion, plasmin and somatic cell proteases are the main origins of endogenous proteolysis [26].

Previous studies have concerned milk composition changes through time after intramammary infusion of 10 μg of LPS. Proteose-peptones (PP), somatic cell count (SCC) and plasmin activity have been followed through time. Two peaks are reported: the first one between 4 and 12 h, concomitant with a high proteolytic activity, involves both plasmin and somatic cells while the second, between 25 and 36 h, occurring with a lower proteolytic activity involves mainly cellular proteolysis [24, 26].

Many works dealing with the substitution in cell culture medium of fetal calf serum (FCS) for whey extracted from milks with low SCC have shown whey positive effects on hybridoma cell culture, such as the increase in antibody production [4, 18]. Freezing storage capacity for at least 2 years was furthermore improved with a higher cell viability [3, 18, 27].

A large number of milk compounds have been reported to have an overwhelming mitogenic activity and to boost cell protein secretion. Among these compounds the key items are the PP fraction, some of the casein fragments resulting from proteolysis and some growth factors whose concentration increases during mastitis [2, 8, 19, 23, 35].

The aim of this work was to test the capacity of mastitic whey to substitute FCS in cell culture medium for hybridomas. With this aim, a LPS experimental mastitis was performed on a cow in order to mimic the three phenomena involved in the inflammatory process without any bacterial interaction. Such milks were characterized beforehand in terms of SCC, plasmin activity and protein composition. In order to evaluate the capacity of mastitic whey for mitogenesis and immunostimulation, a hybridoma cell culture was tested and three criteria were considered for that purpose: viability, growth and antibody production.

2. MATERIALS AND METHODS

2.1. Sampling and milk composition

2.1.1. LPS infusion and sampling

LPS experimental mastitis was performed on a multiparous Holstein cow given an intra-mammary infusion into a single quarter of 10 μg of *E. coli* lipopolysaccharide (LPS 026: B6, Sigma Chemical Co., St. Louis, MO, USA) as described by Moussaoui et al. [26].

For further analyses, two types of milk were considered, normal and mastitic milk. Normal milk corresponded to milk before infusion and mastitic milk to the pool of milks collected at 4, 8 and 12 h post-infusion (PI) corresponding to the highest proteolytic activity according to Moussaoui et al. [26]. The pool was performed with equal volumes of milk in order to have enough milk for cell culture. Raw and skim milks from the infused quarter were frozen at –20 °C for further protein analyses and whey extraction. A low quantity of raw milk (5 mL) was stored at –75 °C for plasmin activity measurements.

Milk composition changes during LPS experimental mastitis, even if variable in intensity, are fairly repetitive (results obtained on 12 cows, data not shown) from one animal to another. A single but representative cow was chosen for the present study for optimal results in terms of milk composition change and especially PP maximum amount.

2.1.2. Milk analysis

Somatic cells were quantified by electronic counting of nuclei after coloration (Fossomatic 5000, Foss Electric, Hillerød, Denmark).

Plasmin activity was measured by a method based on the release of a yellow compound (measured at 405 nm) when a synthetic substrate (D-Val-Leu-Lys p-nitroanilide dihydrochloride; Sigma) that is sensitive to plasmin was hydrolyzed, after addition of dissolving reagent [20].

Total nitrogen, non-protein nitrogen, soluble nitrogen, total casein (tCN) and proteose-peptone contents were determined as described previously [16]. Total protein (TP), soluble protein (SP) and tCN contents were calculated according to the method of Ribadeau-Dumas and Grappin [29]. The pH 4.6 insoluble peptides or IP (including γ-CN) part of tCN was determined with fast protein liquid chromatography (FPLC) with a Mono Q HR 5/5 anion exchange column (Pharmacia, Uppsala, Sweden) according to the method adapted by Le Roux et al. [16].

2.2. Whey preparation

Whey was extracted in acid condition, as mastic milk with a high proteolytic activity could not curdle with rennet.

Whey was filtrated a first time through a 350 μm size filter (Millipore, Paris, France) and then, thanks to a filtration system (Millipore) at several sizes: 8, 1.2, 0.8, 0.45 and 0.22 μm through a membrane (Millipore).

An ultrafiltration (Amicon, Beverly, MA, USA) with 10 000 $g \cdot mol^{-1}$ cut-off was performed first in order to concentrate the whey, to have a final total protein concentration of 20 $g \cdot L^{-1}$ but also to remove small molecules with an inhibitory effect on cell growth [27]. The pH was adjusted to 7.3 with NaOH 5 N and the whey was once again filtrated as described above, then sterilized with two 0.22 μm filtrations in aseptic conditions and thermized for 30 min at 56 °C to inactivate growth inhibitors [27]. The resulting whey fraction, dedicated to cell culture, was subdivided into 5 mL and 10 mL aliquots and stored at –20 °C.

Table I. Concentration of whey and foetal calf serum (FCS) in basal medium used for the adaptation of hybridoma cells to whey medium.

	Day of culture			
	0	2	4	6
Concentration of whey (%)	2.5	5	7.5	9
Concentration of FCS (%)	7.5	5	2.5	1

2.3. Hybridoma cell culture

Anti-hPL hybridoma cells (kindly supplied by Mrs. Capiaumont, Medical Biochemistry Laboratory, faculty of medicine of Nancy, France) were obtained by fusion of a murine Balb/c lymphocyte and murine PAIO type myeloma cell. These cells secreted anti-human placental lactogen hormone IgG1 monoclonal antibodies.

2.3.1. Cell culture

The cells were routinely cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 2 $mmol \cdot L^{-1}$ glutamine (Sigma) and 10% FCS (Sigma), named basic medium, in an incubator at 37 °C, 5% CO_2 and 95% humidity. The cultures were performed in static 25 cm^2 T-flasks (CML, Paris, France).

Three types of media were studied:

– 10% (v/v) FCS (FCS control);

– whey extracted from normal milk, in 9% (v/v) + 1% (v/v) FCS (normal whey or NW);

– whey extracted from mastitic milk, in 9% (v/v) + 1% (v/v) FCS (mastitic whey sample or MW).

Hybridoma cells were progressively adapted to medium containing 9% (v/v) whey and 1% (v/v) FCS. The whey concentration was increased at each change of medium, as shown in Table I. After 10 d of adaptation, the cell growth rate was 80% of that obtained in 10% (v/v) FCS, and their viability was higher than 95%. Cell suspensions were then seeded at a concentration of

2×10^5 cells·mL^{-1} for basic medium supplemented with NW and MW as well as FCS. The cell density of each flask was determined daily using a hemacytometer by staining with 0.5% trypan blue solution; the total suspension was then centrifuged (800 *g*, 5 min at 4 °C). The supernatants were stored at –20 °C for antibody determinations. The whole cells were seeded in flasks containing the same volume of fresh medium. Four repetitions per medium (FCS, NW and MW) were performed. These cultures were carried out over seven days, as beyond this period of time cell viability dropped very quickly until it was below 80%.

2.3.2. ELISA measurements

Antibody production by hybridomas was determined by ELISA: goat IgG anti-mouse IgG (Sigma, St. Louis, USA) was absorbed onto plastic plates (Sigma) overnight at 4 °C. Wells were then rinsed with pH 7.3 PBS reagent and BSA 2% was added overnight at 4 °C. Samples for testing were added at different dilutions and incubated for 2 h at 40 °C. The wells were then rinsed with Tween-PBS 1‰ (v/v) and goat IgG anti-murine IgG labeled with peroxidase (Sigma) was added to each well and incubated for 1 h at 37 °C. Each well was washed with pH 6 phosphate buffer, and peroxydase substrate solution was added (6 mg ortho-phenyl-diamine (OPD) + 12 mL citrate buffer 0.1 mol·L^{-1} + 100 µL H_2O). The reaction was stopped after 30 min, and the absorbance was read at 492 nm. For each antibody sample, the concentration was measured in triplicate: three different wells per sample (FCS, MW and NW) were considered.

2.4. Statistical analyses

Effects of treatment on cell proliferation, cell viability and antibody production/cell were determined by analysis of variance using the repeating measures option of the MIXED procedure of SAS (SAS Inst., Inc., Cary, NC, USA). The model included a fixed factor sampling time and type of complement (FCS, NW and MW). The covariance structure between the different sampling times was defined as being auto-regressive after verification of Akaike and Schwarz-Bayesian criteria [21]. Significance was declared at $P < 0.01$ using the error of the sum of square type III. The values of the analyzed variables were presented as least square means (i.e., adjusted for the effects of the other factors in the model) and least square means were compared with a multiple comparison adjustment.

3. RESULTS AND DISCUSSION

3.1. Milk composition change

3.1.1. Origins of proteolysis

In mastitic milk, SCC was 840 times higher than in normal milk (Tab. II). The SCC value reached for a milk of a quarter infused with 10 µg of LPS was high but similar to previous work, with values greater than 4×10^6 c·mL^{-1} between 8 and 36 h post-injection [24, 26]. This observation illustrated an acute inflammatory process with an increase in the permeability of the epithelial barrier as somatic cells came from the blood stream [28]. These somatic cells, where the polymorphonuclear neutrophils (PMN) are predominant, have a major role in endogenous proteolysis, and particularly in caseinolysis [1, 26, 34].

Plasmin activity in mastic milk increased 3.6 times more than in normal milk (Tab. II). The increase of plasmin activity could be explained by an increased influx of plasminogen from blood to milk [6, 11] with its activation into plasmin possibly mediated by somatic cells [6, 9, 11, 33].

3.1.2. Milk protein composition

tCN content in mastitic milk was 0.8 times lower than in normal milk (Tab. II).

Table II. Milk composition change of the infused quarter, comparison of normal milk (before infusion) with mastitic milk (pool of times 4, 8 and 12 h) after lipopolysaccharide intramammary infusion to a dairy cow (n = 1). For each parameter, the variation between normal and mastitic milk has been calculated.

Parameter	Normal milk	Mastitic milk	Variation
SCC (10^6 c·mL^{-1})	0.01	8.4	× 840
PA (μmol of *p*-nitroanilide per L per h)	11.7	42.3	× 3.6
tCN (g·L^{-1})	25.7	20.8	× 0.8
pH 4.6 IP (%)	2.1	7.2	× 3.4
PP (g·L^{-1})	0.94	2.4	× 2.5
SP/TP	0.13	0.27	× 2.1

SCC = somatic cell count; PA = plasmin activity; tCN = total casein; IP = insoluble peptides; PP = proteose-peptones; SP = soluble proteins; TP = total proteins.

This decrease was the consequence of two simultaneous mechanisms: a lower activity of synthesis by the mammary gland epithelial cells as well as an increase in endogenous proteolysis [16, 28]. The second mechanism leads to a transfer of a part of the casein fraction to the soluble fraction (pH 4.6 insoluble peptides, PP and SP) [16].

PP content, percentage of pH 4.6 insoluble peptides and the ratio SP/TP were increased by a factor of 2.5, 3.4 and 2.1, respectively, in comparison with normal milk (Tab. II). PP contained many components resulting from CNs degradation [17]. Thus, PP and γ-CNs are commonly considered as good indicators of endogenous proteolysis by many authors [16, 32]. The increase in the SP part of TP in milk could be explained by the permeability of the blood-milk barrier with a massive influx of bovine serum albumin and immunoglobulins [7, 30] as well as some peptides resulting from α-lactalbumin (α-LA) and β-lactoglobulin degradation by cellular proteases [10].

3.2. Hybridoma cell culture

3.2.1. Cell proliferation

For all modalities (FCS, MW and NW), cell density was adjusted at 2×10^5 c·mL^{-1} at day 0 in order to test only the medium-effect (Fig. 1A).

For FCS control, cell proliferation increased significantly ($P < 0.01$) by a factor of 12 within 6 d, reaching a maximum of 2.36×10^6 c·mL^{-1}. Cell density plateaued after day 6.

For whey samples, proliferation increased significantly until day 6 reaching a maximum of 1.8×10^6 c·mL^{-1} for the normal whey and 9.6×10^5 c·mL^{-1} for mastitic whey, thereby multiplying the original proliferation by a factor of 9 and 5, respectively.

Cell proliferation dropped for whey samples beyond day 6. A maximum was observed at day 6 for FCS and whey control while Legrand et al. [18] noticed a maximum at day 4 with sweet whey followed by a quick drop of living cells. In our study, acid whey was used even though sweet whey provides better results for cell

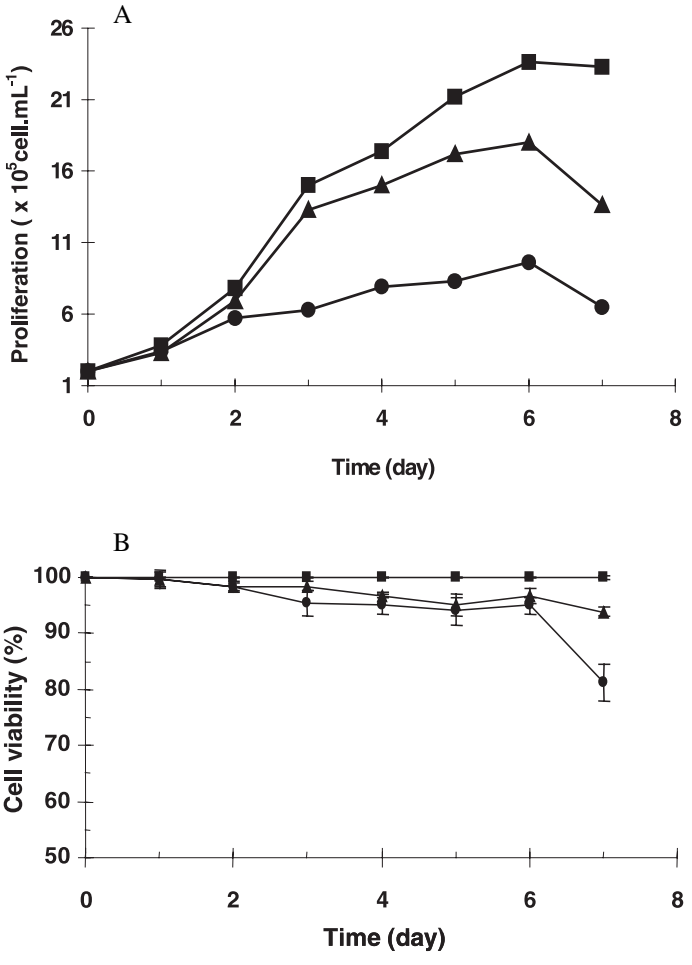

Figure 1. Hybridoma cell proliferation (A) and viability (B). Cells were seeded at 2×10^5 c·mL^{-1} in RPMI-1640 medium supplemented by fetal calf serum (FCS, ■), normal whey + 1% FCS (▲) or mastitic whey + 1% FCS (●). When not visible, error bars are contained within the symbols.

culture [27]. Thus, milks with very high SCC and indeed with a great caseinolysis could not curdle with rennet, which made necessary the use of very acidic conditions.

The results observed with mastitic whey obtained after an LPS infusion suggest that some components with an inhibitory effect on cell proliferation and a decreasing mitogenic activity would appear in such whey. These components were not lethal, as cell proliferation did not drop suddenly after the seeding of the cells in the medium. On the other hand, the hydrophobic fraction of PP has been reported to have a mitogenic effect on hybridoma cell culture when extracted from normal milk characterized by a low SCC [23]. The high proteolytic activity could have altered this mitogenic property, as plasmin has already been reported to degrade PP [12, 13].

3.2.2. Cell viability

Cell viability in the FCS control medium averaged 100% until day 6. It reached 95% with normal whey during the same period. Beyond day 6, viability dropped significantly ($P < 0.01$), which was more pronounced for mastitic whey (Fig. 1B).

Results obtained by Legrand et al. [18] showed a cell viability of 100% with FCS and sweet whey till day 2. Beyond day 4 viability was less than or equal to 80%, which showed that whey extracted from milk with very high SCC was able to keep alive hybridoma cells (viability greater than 95%) for 6 d.

According to the cell proliferation results, FCS appeared to have a positive action on viability as well as on cell proliferation. The same conclusions could be inferred for normal whey according to Legrand et al. [18] on short-term observations. Normal whey as well as FCS, had a mitogenic activity, which confirmed that such whey was able to substitute to FCS, that is commonly used to supplement basic cell culture medium. Whey, however, has to be supplemented with a minimum of 0.5% (v/v) FCS and for optimal results with 1% (v/v) FCS in the case of a long-term (over 3 d) cell culture [27]. The use of 1% (v/v) FCS in our study permitted us to maintain cell viability high enough over 3 d in order to evaluate the capacity of whey (normal and mastitic) to promote cell proliferation and antibody production. In addition, basic medium supplemented with 1% FCS only has previously been tested; a very low cell proliferation was observed, as low as with 10% whey [27].

3.2.3. Antibody production

An antibody concentration was determined on cell culture medium (containing medium supplemented with FCS, NW or MW) as well as on FCS, NW and MW without any addition of basal medium. No crossing reactions between murine and bovine IgG have been previously reported; consequently only bovine IgG secreted by hybridomas were measured.

For FCS, antibody production increased significantly ($P < 0.01$) from day 1 and reached a maximum at day 3 (2 times the concentration at day 1). A steady state was noticed after 3 d. The same pattern was observed for normal whey with, however, an increase of 1.5 times. For mastitic whey, antibody concentration reached a maximum at day 2 with a slight decrease at day 3, the steady state observed afterwards was, however, 1.4 times higher than FCS and 1.1 times higher than NW (Fig. 2A). In our study, antibody production during the exponential growth phase of hybridomas plateaued, especially for mastitic whey. This phenomenon was more pronounced with the antibody/cell ratio study that allowed a more relevant comparison between the three media types regarding the antibody production per cell (Fig. 2B). MW medium showed an overwhelming difference compared with NW and FCS media. Antibody production for MW compared with FCS was twice as high at days 2 and 3 while it was three times as high at days 4, 5 and 6.

Thus, MW showed a lower mitogenic activity on hybridoma cell culture, but at the same time a great stimulation of Igs production. NW and FCS showed very close proliferation and antibody production results that could make us infer that one or several new compounds might be produced during endogenous proteolysis resulting from the inflammatory process. The PP fraction would be intimately linked to these observations as its composition changes qualitatively with the apparition of several fragments of CN, which takes place only during the acute phase of inflammation corresponding also to the highest period of proteolysis [25].

The main explanation of IgG production would be an increase in IL6 concentration, which is reported to be up to 11 times greater in milk of infused quarters after a

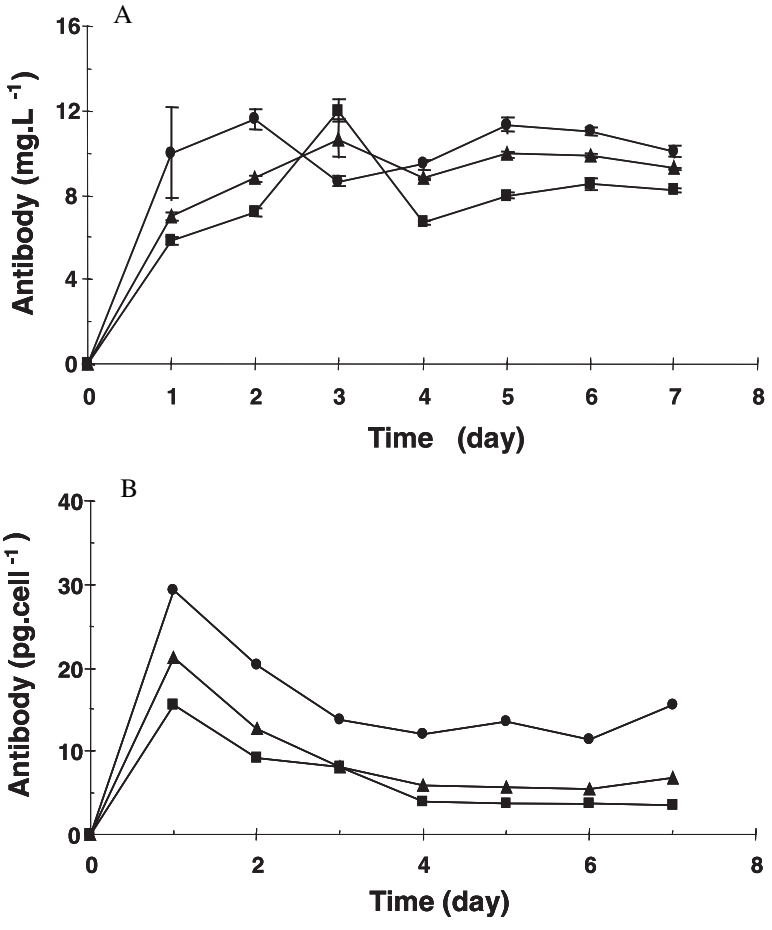

Figure 2. Antibody production, expressed in absolute values (A) and per cell (B), of hybridoma cells cultured in RPMI-1640 medium supplemented with fetal calf serum (FCS, ■), normal whey + 1% FCS (▲) or mastitic whey + 1% FCS (●). When not visible, error bars are contained within the symbols.

10 μg intramammary infusion into the cow, from 2.5 h post-injection [31]. The immunostimulating effect is not proportional to IL6 concentration. Indeed, when the IL6 concentration is multiplied by 10, that of Igs increases from 0.75 $\mu g \cdot mL^{-1}$ to 0.79 $\mu g \cdot mL^{-1}$; for an increase of 100 times in IL6 concentration, that of Igs increases from 0.75 $\mu g \cdot mL^{-1}$ to 0.76 $\mu g \cdot mL^{-1}$. These results also report the existence of a threshold; over a certain concentration of IL6, the production of Igs becomes stable [14]. The immunostimulating effect observed with MW could also not be explained by the increase in IL6 concentration. Other explanations could be more related to caseinolysis and to the generation of some peptides with a biological property. Thus, para-κ-CN, which results from κ-CN cleavage by cathepsin D [15] is reported to stimulate antibody production [35]. Furthermore, two fragments of β-CN

(f63-68 and f191-193), one from α_{s1}-CN (f194-199) and a fragment of α-LA (f51-53) have been reported to be immunomodulating peptides [5].

In addition, some components of the PP fraction are strongly linked to proteolysis (e.g. β-CN1P (f29-105 and f29-107) and β-CN5P (f1-105 and f1-107)) with an increasing concentration during mastitis [17]. Some of these compounds could have a biological activity.

4. CONCLUSION

Experimental mastitis in cow induced with LPS involves an important change in milk composition, especially in the protein part [26].

Whey from milk with high SCC and also with greater proteolysis had a low mitogenic activity but a greater immunostimulating effect. Basic medium supplemented with such whey revealed a better efficiency with a greater antibody production per cell. The decrease in mitogenic activity would, however, be explained by the degradation of the hydrophobic fraction of PP, and the immunostimulating effect would be linked to new CN fragments in the PP fraction.

ACKNOWLEDGMENTS

We thank Professor P. Nabet, J. Capiaumont, C. Claudon and M. Marie for their scientific cooperation. We acknowledge, furthermore, the excellent technical assistance provided by C. Grandclaudon, C. Hognon and T. Fontaine.

REFERENCES

[1] Andrews A.T., Breakdown of caseins by proteinases in bovine milks with high somatic cell counts arising from mastitis or infusion with bacterial endotoxin, J. Dairy Res. 50 (1983) 57–66.

[2] Bruckmaier R.M., Schallibaum M., Blum J.W., *Escherichia coli* endotoxin induced mastitis in dairy cows: changes and importance of insulin-like-growth factor and oxytocin, Milchwissenchaft 48 (1993) 374–378.

[3] Capiaumont J., Jacob C., Frossi C., Nabet P., Dousset B., Whey: a byproduct for eucaryotic cell cryopreservation, In Vitro Cell. Dev. Biol. Anim. 36 (2000) 631–632.

[4] Derouiche A.F., Legrand C., Bour J.M., Capiaumont J., Gelot M.A., Dousset B., Belleville F., Nabet P., Linden G., Biochemical aspects of whey fraction capable of promoting hybridoma proliferation. Comparison with fetal calf serum, Lait 70 (1990) 313–324.

[5] Fiat A.-M., Migliore-Samour D., Jollès P., Drouet L., Bal dit Sollier C., Caen J., Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities, J. Dairy Sci. 76 (1993) 301–310.

[6] Grufferty M.B., Fox P.F., Milk alkaline proteinase, J. Dairy Res. 55 (1988) 609–630.

[7] Guidry A.J., Ost M., Mather I.H., Shainline W.E., Weinland B.T., Sequential response of milk leucocytes, albumin, immunoglobulins, monovalent ions, citrate and lactose in cows given infusions of *Escherichia coli* endotoxin into the mammary gland, Amer. J. Vet. Res. 44 (1983) 2262–2267.

[8] Guimont C., Marchall E., Girardet J.-M., Linden G., Biologically active factors in bovine milk and dairy byproducts: influence on cell culture, Crit. Rev. Food Sci. Nutr. 37 (1997) 393–410.

[9] Heegaard C.W., Rasmussen L.K., Andreasen P.A., The plasminogen activation system in bovine milk: differential localization of tissue-type plasminogen activator and urokinase in milk fractions is caused by binding to casein and urokinase receptor, Biochem. Biophys. Acta 1222 (1994) 45–55.

[10] Jakobsson I., Borulf S., Lindberg T., Benediktsson B., Partial hydrolysis of cow's milk proteins by human trypsins and elastases in vitro, J. Pediatr. Gastroenterol. Nutr. 2 (1983) 613–616.

[11] Kaartinen L., Veijalainen K., Kuosa P.L., Sandholm M., Endotoxin-induced mastitis: inhibition of casein synthesis and activation of the caseinolytic system, J. Vet. Med. B 35 (1988) 353–360.

[12] Kanno C., Ogawa H., Proteolysis of lactophorin in skim milk by bovine milk indigenous proteinases, Jpn. J. Zootech. Sci. 60 (1989) 797–864.

[13] Kanno C., Ogawa H., Proteolytic degradation of lactophorin from bovine milk by plasmin and trypsin, Jpn. J. Zootech. Sci. 60 (1989) 865–873.

[14] Kawahara H., Maeda-Yamamoto M., Hakamata K., Effective induction and acquisition of human monoclonal IgE antibodies reactive with house-dust mite extract, J. Immunol. Meth. 233 (2000) 33–40.

[15] Larsen L.B., Benfeldt C., Rasmussen L.K., Petersen T.E., Bovine milk procathepsin D and cathepsin D: coagulation and milk degradation, J. Dairy Res. 63 (1996) 119–130.

[16] Le Roux Y., Colin O., Laurent F., Proteolysis in samples of quarters milk with varying somatic cell counts. 1. Comparison of some indicators of endogenous proteolysis in milk, J. Dairy Sci. 78 (1995) 1289–1297.

[17] Le Roux Y., Girardet J.M., Humbert G., Laurent F., Linden G., Proteolysis in samples of quarters milk with varying somatic cell counts. 2. Component PP3 and β-casein-1P (f29-105 and f29-107) of the proteose-peptone fraction, J. Dairy Sci. 78 (1995) 1298–1305.

[18] Legrand C., Capiaumont J., Belleville F., Nabet P., Comparison of metabolism of hybridoma cells cultured in media supplemented with whey or fetal calf serum, Biotechnol. Progr. 9 (1993) 573–579.

[19] Liebe A., Schams D., Growth factors in milk: interrelationships with somatic cell count, J. Dairy Res. 65 (1998) 93–100.

[20] Linden G., Humbert G., Kouomegne R., Guimgamp M.F., Reagent for rendering biological fluids transparent and its analytical applications, Eur. Patent Appl. EP 0 246 978 B1 (1987), 10 p.

[21] Little R.C., Milliken G.A., Stroup W.W., Wolfinger R.D., SAS system for mixed models, SAS Institute Inc., Cary, NC, USA, 1996, 633 p.

[22] Massart-Laën A.M., Burvenich C., Massart D.L., Triacylglycerol fatty acid composition of milk from periparturient cows during acute *Escherichia coli* mastitis, J. Dairy Res. 61 (1994) 191–199.

[23] Mati A., Moulti-Mati F., Girardet J.M., Fokou E., Belleville-Nabet F., Nabet P., Linden G., Mitogenic activity of hydrophobic fractions of proteose-peptone from cows', ewes', goats' milk measured with MARK 3 hybridoma culture, J. Dairy Res. 60 (1993) 443–448.

[24] Michelutti I., Le Roux Y., Rainard P., Poutrel B., Laurent F., Sequential changes in milk protein composition after experimental *Escherichia coli* mastitis, Lait 79 (1999) 535–549.

[25] Moussaoui F., Le Roux Y., Laurent F., Origines de la protéolyse dans les laits d'une mammite expérimentale à LPS, in: 51th annual meeting of the European association for animal production, Wageningen Pers, The Hague, The Netherlands, 6 (2000) 224–225.

[26] Moussaoui F., Michelutti I., Le Roux Y., Laurent F., Mechanisms involved in milk endogenous proteolysis induced by a lipopolysaccharide experimental mastitis, J. Dairy Sci. 85 (2002) 2562–2570.

[27] Nabet P., Belleville-Nabet F., Linden G., Les peptides du lait à activités physiologiques. I. Facteurs de croissance dans le lait et le lactosérum, Lait 71 (1991) 225–239.

[28] Nickerson S.C., Pankey J.W., Neutrophils migration through "teat and tissues" of bovine mammary quarters experimentally challenged with *Staphylococcus aureus,* J. Dairy Sci. 67 (1984) 826–834.

[29] Ribadeau-Dumas B., Grappin R., Milk protein analysis, Lait 69 (1989) 357–372.

[30] Shuster D.E., Harmon R.J., Jackson J.A., Hemken R.W., Suppression of milk production during endotoxin-induced mastitis, J. Dairy Sci. 74 (1991) 3763–3774.

[31] Shuster D.E., Kehrli Jr. M.E., Sevens M.G., Cytokine production during endotoxin-induced mastitis in lactating dairy cows, Amer. J. Vet. Res. 54 (1993) 80–85.

[32] Urech E., Puhan Z., Schällibaum M., Changes in milk protein fraction as affected by subclinical mastitis, J. Dairy Sci. 82 (1999) 2402–2411.

[33] Verdi R.J., Barbano D.M., Effect of coagulants, somatic cell enzymes, and extracellular bacterial enzymes on plasminogen activation, J. Dairy Sci. 74 (1991) 772–782.

[34] Verdi R.J., Barbano D.M., Properties of proteases from milk somatic cells and blood leukocytes, J. Dairy Sci. 74 (1991) 2077–2081.

[35] Yamada K., Ikeda I., Nakajima H., Shirahata S., Murakami H., Stimulation of proliferation and immunoglobulin production of human-human hybridoma by various types of caseins and their protease digests, Cytotechnology 5 (1991) 279–285.