

Concentration of human milk immunoglobulins by ultrafiltration

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Summary — Immunoglobulin fractions have been successfully used *in vivo* for the treatment of illnesses such as chronic diarrhea in the newborn. When used to fortify human breast milk, it is important that the milk immunoglobulins remain unaltered during the protein concentration process. The purpose of the present study was to evaluate changes in the quantity of IgA, IgG and IgM immunoglobulins after concentration of human milk proteins by an ultrafiltration membrane technique. Immunoglobulins present in human milk and the retentate obtained after ultrafiltration of the milk were analyzed by ELISA and gel filtration. The recovery of immunoglobulins in the retentate obtained from ultrafiltration was high: 100%, 70% and 60% of the total IgA, IgG and IgM, respectively. ELISA showed that IgA was the major immunoglobulin present in human milk. Gel filtration analysis showed that the IgA immunoglobulins were entirely in the form of dimeric secretory IgAs both in the milk and in the retentate. Ultrafiltration appears to be a useful technique for preparing immunoglobulin-enriched human milk fractions.

ultrafiltration / human milk / immunoglobulins

Résumé — Concentration, par ultrafiltration, des immunoglobulines dans le lait humain. Les immunoglobulines du lait humain ont été utilisées avec succès comme traitement de certaines pathologies chez le nouveau-né. Le travail présenté dans ce manuscrit a été entrepris pour suivre les concentrations en immunoglobulines dans un lait humain au cours d'une ultrafiltration qui a pour but de concentrer la fraction protéique du lait. Les immunoglobulines présentes dans le lait humain et dans le rétentat obtenu par ultrafiltration sont dosées par une technique ELISA avant et après chromatographie en gel filtration. Les proportions des immunoglobulines retrouvées dans le rétentat après ultrafiltration sont respectivement de 100%, 70% et 60% des IgA, IgG et IgM totales du lait de départ. Le dosage ELISA montre que les IgA ($0,274 \pm 0,04$ g/kg) sont les immunoglobulines majoritaires dans le lait humain. Les chromatographies en gel filtration révèlent que les IgA du lait et du rétentat sont sous forme dimérique (IgA sécrétoires). L'ultrafiltration, qui ne détruit pas les immunoglobulines du lait, semble donc une bonne technique pour préparer des fractions de lait humain enrichi en immunoglobulines.

ultrafiltration / lait humain / immunoglobulines

INTRODUCTION

Human milk is still considered to be the best tolerated form of milk for the newborn infant (Ogra and Greene, 1982), but is nutritionally inadequate for the very low birth-weight neonate who requires more protein than is available in human breast milk for the catch-up growth phase characteristic of these infants. For this reason, human milk protein concentrates have been developed for the fortification of breast milk (Lucas *et al*, 1980; Voyer *et al*, 1984). Human milk is also known to contain protective factors against infection and disease in the newborn (Pitt, 1979), including immunoglobulins. For example, a bacteriostatic effect of milk protein fractions fortified in secretory immunoglobulin A and/or lactoferrin has been demonstrated *in vitro* (Bullen *et al*, 1972; Spik *et al*, 1978; Arnold *et al*, 1980). Such protein fractions have also been used with success *in vivo* in the treatment of neonatal diarrhea (Navarro *et al*, 1983; Spik *et al*, 1984).

Therefore, it is possible that fortifying human breast milk with human milk protein fractions could not only be useful in the neonatal unit for the feeding of very low birthweight neonates, but also in gastroenterology and pediatric surgery units where it could serve as a potential therapeutic aid. In regard to the latter, it is important that the milk immunoglobulins remain unaltered after protein concentration. The purpose of the present study was to evaluate changes in the quantity of immunoglobulins A, G and M (IgA, IgG, IgM) after concentration of human milk proteins by an ultrafiltration membrane technique (Maubois and Brulé, 1982; Maubois, 1984).

MATERIALS AND METHODS

Milk collection and ultrafiltration

Raw pooled human milk (RPM), provided by the Lactarium de Lille (Lille, France), was collected from healthy mothers. The milk was kept frozen at -18°C up until the time of use at which point 313.8 kg was thawed, skimmed and ultrafiltered (fig 1) in the Laboratoire de Recherches de Technologie Laitière (INRA, Rennes, France) according to the technique of Maynard *et al* (1989). Thirty-six kg of retentate was obtained from the ultrafiltration.

After 2 successive centrifugations of the RPM (1 600 g, 4°C , 30 min), the fat layer was removed. The defatted RPM and retentate were then ultracentrifuged at 100 000 g for 2 h at 4°C . The supernatants were aspirated and aliquoted, whereas the pellets (which contain casein) were discarded. The total immunoglobulin response was quantitatively recovered in the supernatant according to ELISA.

Fast protein liquid chromatography

Fast protein liquid chromatography (FPLC) was performed with an LCC 500 system (Pharmacia, Saint-Quentin-en-Yvelines, France) equipped with a Superose 12 (Pharmacia) gel filtration column and a UV detector set at a wavelength of 280 nm. The column was eluted with a 50 mM PBS buffer (pH 7.0) at a flow rate of 0.4 ml/min. After filtration through a $0.22\text{-}\mu\text{m}$ filter, 200 μl of each sample were injected into the column and the eluent was collected in 0.8-ml fractions.

Enzyme linked immunosorbent assay (ELISA)

For the ELISA of IgA, IgG and IgM, microtiter plates for micro-ELISA (Nunc) were used as the

MILK PREPARATION

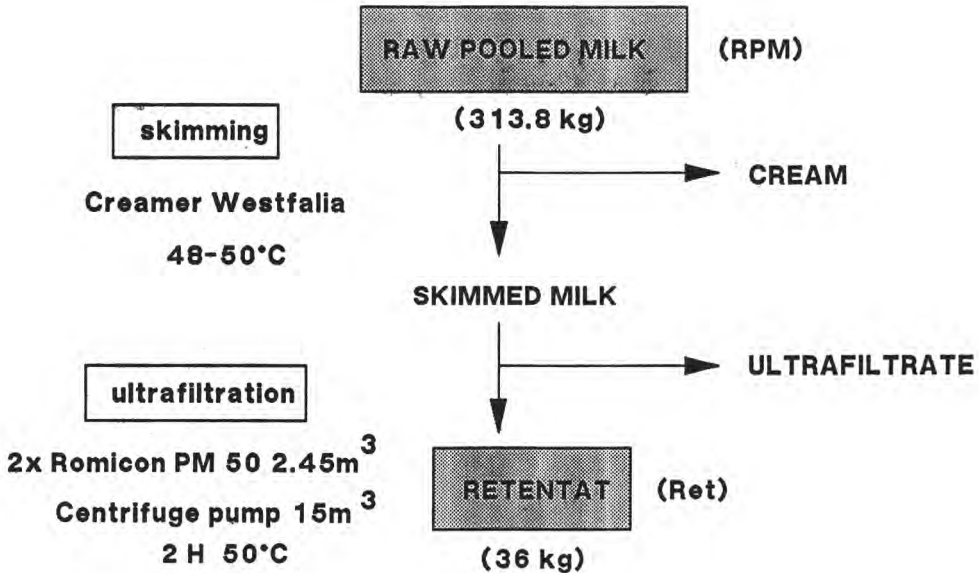


Fig 1. Preparation of 36 kg retentate from 313.8 kg raw pooled milk by the ultrafiltration technique. *Schéma de préparation de 36 kg de rétentat obtenu par ultrafiltration de 313,8 kg d'un mélange de lait cru humain (RPM).*

solid-phase carrier. Goat antibody anti-human IgG (Biosys), anti-human IgM (Miles) and anti-human IgA (Behring) were applied at 100 μ l/well in a coupling buffer (60 mM bicarbonate buffer, pH 9.8) at dilutions of 1/2 000, 1/1 500, and 1/2 000, respectively. After an overnight incubation at 4 °C, plates were washed in PBS-Tween buffer (phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20). Test samples were then incubated in duplicate in 100 μ l/well PBS-Tween at 37 °C for 90 min. After washing with PBS-Tween buffer, plates were incubated with goat antibody peroxidase conjugate (100 μ l/well) at 37 °C for 1 h more. Anti-IgA (Biosys), anti-IgG (Biosys) and anti-IgM (Miles) were previously diluted in PBS-Tween at dilutions of 1/3 000, 1/6 000 and 1/4 000, respectively. After the final wash, peroxidase activity was detected with 100 μ l/well orthophenylenediamine substrate (Prola-

bo) freshly dissolved at 0.4 mg/ml in citrate buffer (50 mM, pH 5.0). After 15 min, the reaction was stopped by addition of 5 N sulfuric acid (50 μ l/well), and quantified by measurement of absorbance at a wavelength of 490 nm on a microplate autoreader (Bio-Tek instrument). Standard curves for IgA, IgM and IgG were established with a standard (Behring, Rueil-Malmaison, France).

RESULTS AND DISCUSSION

The concentration of IgG, IgM and IgA was measured by ELISA both in the RPM and the retentate obtained after ultrafiltration (table I). IgA was the major immunoglobu-

Table 1. IgG, IgM, and IgA ELISA measurements in the raw pooled human milk (RPM) and retentate after ultrafiltration ($n = 6$).*Détermination des IgG, IgM et IgA par technique ELISA dans un lait humain (RPM) et son rétentat obtenu par ultrafiltration ($n = 6$).*

	Raw pooled milk	Retentate	Concentration yield	Total recovery (%)
Total weight (kg)	313.8	36	—	—
$N \times 6.38^a$ (g/kg)	14.56	63.75	4.4	50.2
IgG ^b (g/kg)	0.024 ± 0.0008	0.147 ± 0.019	6.1	69.8
IgM ^b (g/kg)	0.012 ± 0.0006	0.061 ± 0.002	4.9	58.3
IgA ^b (g/kg)	0.274 ± 0.043	2.46 ± 0.23	8.9	100

^a Total nitrogen content was determined by the micro-Kjeldahl technique (Hambraeus *et al*, 1976). ^b ELISA measurements in samples after ultracentrifugation of RPM and retentate.

lin present in the human breast milk. After ultrafiltration, the Ig fractions remained in the retentate with a 5–9 concentration yield relative to the RPM. Furthermore, 100, 70 and 60% of the total IgA, IgG and IgM respectively was recovered in the retentate.

Chromatography of the supernatants obtained from the ultracentrifugation of RPM and of the retentate was performed by gel filtration (figs 2, 3). In order to differentiate dimeric secretory IgA (IgAs) from monomeric IgA, the collected fractions were analysed by ELISA. A standard serum (Behring), which contained both monomeric IgA (160 000 Da) that eluted at 8.8 ml and dimeric secretory IgA (420 000 Da) that eluted at 4.8 ml, was used as the standard (fig 2A). Under the same conditions, IgA ELISA monitoring of the gel filtration fractions from RPM (fig 2B) and retentate (fig 2C) showed only one peak of IgAs eluted at 4.8 ml. Monitoring of RPM and retentate at an absorbance of 280 nm indicated a major peak of α -lactalbumin

(14 000 Da) eluted at 12 ml. IgG and IgM ELISA monitoring of the gel filtration fractions indicated that both IgG and IgM were eluted in single peaks at 10 ml and 6 ml, respectively, in the raw pooled milk (fig 3A) and retentate (fig 3B).

The current work confirms that IgA is the major Ig in human milk and is present entirely in a dimeric secretory form (Hanson *et al*, 1973; McClelland, 1982; Woodhouse *et al*, 1988). A significant amount of the Ig fraction of human milk can be recovered in the retentate obtained after ultrafiltration of the milk. IgA is recovered most efficiently from the retentate where it remains in its dimeric secretory form. These results indicate that ultrafiltration could be a useful technique for the preparation of Ig-enriched milk fractions. However, the conditions allowing efficient sterilization of such a product without alteration of the Ig fraction need to be established before it can be used as a therapeutic aid in the treatment of illness in very low birthweight neonates.

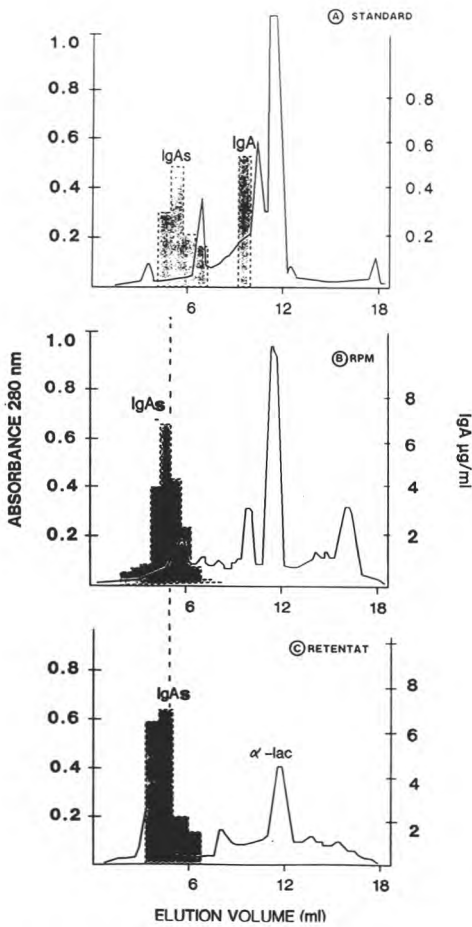


Fig 2. Profile of the IgA concentrations (---), as determined by ELISA, of the FPLC gel filtration fractions of (A) standard, (B) raw pooled milk and (C) retentate on Superose 12 (—). In comparison to the standard in which there were 2 forms of IgA (monomeric and dimeric), only dimeric IgA (IgAs) was found in the RPM and retentate samples.

Profil des concentrations en IgA (---) dosées par technique ELISA après chromatographie FPLC sur colonne Superose 12 (—) d'un témoin standard (A), du lait cru (B) et du rétentat (C). D'après le profil standard (A), on ne retrouve que des IgA de sécrétion dans le lait cru (RPM) et le rétentat.

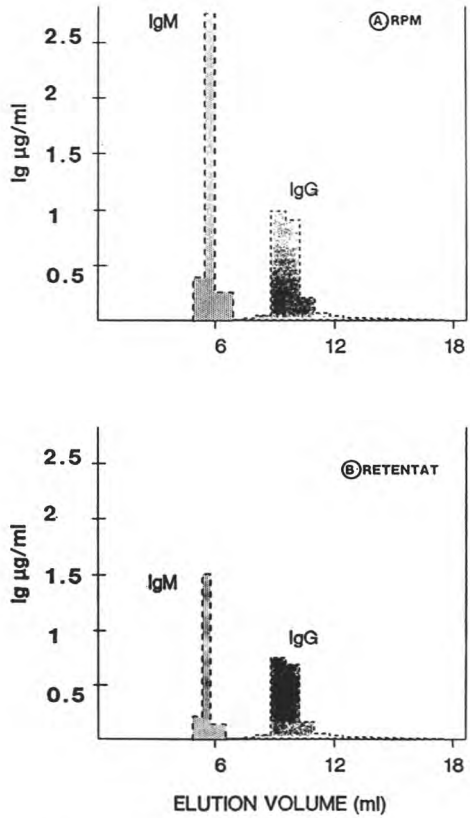


Fig 3. Profile of the IgG and IgM concentrations, as determined by ELISA, of the FPLC gel filtration fractions of (A) raw pooled milk and (B) retentation Superose 12.

Profil des concentrations en IgG et IgM dosées par technique ELISA après chromatographie FPLC sur colonne Superose 12 du lait cru (A) et du rétentat (B).

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