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# Characterization of an antithrombotic peptide from κ-casein in newborn plasma after milk ingestion

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**Bovine and human κ-caseinoglycopeptides, two antithrombotic peptides derived from the corresponding κ-caseins, were detected in physiologically active concentrations in the plasma of 5-d-old newborn infants after ingestion of cow's-milk-based formula or human milk respectively. It is suggested that these two bioactive peptides are released from milk proteins during digestion.**

**Casein: Antithrombotic peptides: Newborn infants**

Historically, milk proteins have been considered only as food proteins for young mammals because they are their principal source of amino acids. They might also have a physiological importance as a source of biologically active peptides. Since 1979, numerous peptides whose sequences and locations are known have been shown to possess various activities including opiate, antihypertensive, immunomodulator, antibacterial, anti-aggregating and antithrombotic effects (Fiat *et al.* 1993).

We have characterized a series of peptides from κ-casein which have platelet-function activity. A natural and synthetic undecapeptide from bovine κ-casein (MAIPPKKNQDK, residues 106–116) inhibited ADP-induced platelet aggregation and fibrinogen binding in a concentration-dependent manner (Jollès *et al.* 1986). Peptides from bovine κ-casein showed antithrombotic activity in the guinea-pig; these included the undecapeptide itself, the pentapeptide (KNQDK, residues 112–116) which is the sequence of minimal activity, and the caseinoglycopeptide (cCGP; residues 106–169) which contains the undecapeptide (C. Bal dit Sollier, personal communication). Human κ-caseinoglycopeptide (hCGP) also includes an antithrombotic peptide which is more potent than that from the cow (cCGP; C. Bal dit Sollier, personal communication).

In the calf's stomach, during the first phase of the digestion, the main peptide released from bovine casein was the κ-caseinoglycopeptide (Yvon & Pélissier, 1987). Moreover, there is now irrefutable evidence that small amounts of intact peptides and proteins enter the circulation under normal circumstances after ingestion (Gardner, 1988).

The aim of the present study therefore, was to identify κ-caseinoglycopeptides in the plasma of newborn infants exclusively breast-fed or fed only on a cows'-milk-based formula, in order to characterize and quantify these peptides.

## MATERIALS AND METHODS

### *Subjects*

The present study was performed on twelve healthy, full-term, 5-d-old infants. The protocol was approved by the Ethics Committee (Lariboisière–Fernand Widal–Saint Lazare Hospitals, Paris, France). Written consent was obtained from each parent. Neither infants nor mothers had been prescribed drugs.

Six newborn infants were exclusively breast-fed ($B_1$, $B_2$, $B_3$, $B_4$, $B_5$, $B_6$). Six other infants were exclusively fed on a cows'-milk-based formula: five infants received 'Nidal first age' (Nestlé, Courbevoie, France; $F_{N1}$, $F_{N2}$, $F_{N3}$, $F_{N4}$, $F_{N5}$) and one infant received 'Alma first age' (Jacquemaire santé, Levallois, France; $F_{A1}$).

### *Blood sampling*

With the approval of the Ethics Committee, blood was collected on the fifth day of age at the time of the Guthrie test. Standard plasma was collected from fasted infants or adults. Venous blood (1–1·5 ml) was collected from the arm (9 vol. blood: 1 vol. 0·13 mM-trisodium citrate). Blood was withdrawn rapidly to prevent coagulation. It was centrifuged at 3000 rev./min for 5 min and the plasma frozen at −20° before analysis. For HPLC the plasma was treated with perchloric acid (final concentration 166 μM), centrifuged again at 10000 rev./min for 10 min at room temperature and the supernatant fraction chromatographed.

### *Separation and identification of peptides by HPLC*

Analyses were performed using Gilson HPLC equipment (Gilson Medical Electronics, Villiers-Le-Bel, France). A portion (100 μl) of treated plasma was applied to a RP 300 reverse-phase column (4·6 × 220 mm; Brownlee Labs, San José, CA, USA). The flow-rate was 1 ml/min and peptides were detected at 220 nm. The solvents used in the gradient were trifluoracetic acid (TFA) aqueous solution (1 ml/l; solvent A) and acetonitrile (Baker Chemicals, Deventer, Holland) containing TFA (1 ml/l; solvent B). Two gradients, C and H, were used (Table 1).

### *Amino acid sequence analysis*

The N-terminal amino acid sequence of the peptides was determined by Edman degradation using an automated gas-phase sequencer (model 470 A; Applied Biosystems, Foster City, CA, USA). The phenylthiohydantoin amino acid derivatives were identified automatically using an Applied Biosystems 120 A phenylthiohydantoin amino acid derivatives analyser used on-line with the sequencer 470 A.

### *ELISA*

Microtitre plates (Maxisorb; NUNC, Copenhagen, Denmark) were used and shaken on a Vari-shaker (Dynatech Laboratories, Alexandria, VI, USA). Absorbance was read on a microplate reader (model EL 308 EIA Bio-tek; Osi, Paris, France). All antigens and samples were diluted in 20 mM-phosphate-buffered saline (PBS) with 0·15 M-NaCl, pH 7·5 (buffer A) and the antisera in 20 mM-PBS with 0·15 M-NaCl, pH 7·5 containing Tween 20 (0·5 ml/l; buffer B). Plates were washed with buffer B. The peroxidase (*EC* 1.11.1.7) substrate (2·2'-azino-bis (3-ethylbenzthiazoline-6-sulphonate); ABTS; 0·8 mg/ml) was mixed with 10 nM-$H_2O_2$ in a 0·1 M-$Na_2HPO_4$ and 0·04 M-citric acid solution, pH 5·6.

Rabbit cCGP antisera were raised by subcutaneous injection of cCGP (1 mg) in 0·5 ml saline solution emulsified with 0·5 ml Freund's complete adjuvant. Similar booster injections were made at 2-week intervals using incomplete Freund's adjuvant. The rabbits

Table 1. *C and H elution gradients for HPLC used to detect bovine (c) and human (h) κ-caseinoglycopeptides (CGP) respectively*

| | Elution time (min) | Volume of solvent B in Solvent A* (ml/l) |
|---|---|---|
| C gradient | 0 | 0 |
| | 10 | 0 |
| | 15 | 150 |
| | 85 | 500 |
| | 91 | 1000 |
| H gradient | 0 | 0 |
| | 10 | 0 |
| | 100 | 1000 |

* For details of composition, see p. 584.

were bled 8 d after the sixth booster challenge. The specificity of the antibodies was controlled by double immunodiffusion.

The conjugate, goat anti-rabbit peroxidase-labelled immunoglobulin (IgG (H+L) fraction), was obtained from Diagnostics Pasteur, Marne-la-Coquette, France. cCGP and bovine κ-casein were prepared in our laboratory according to the procedures of Jollès *et al.* (1961) and McKenzie *et al.* (1961) respectively.

cCGP was quantified by competitive ELISA. Each well of the microtitre plate was coated with κ-casein (2·5 μg/ml; 200 μl) and incubated for 2 h at 37°. Plates were washed three times and 200 μl bovine serum albumin solution (10 g/l) was added. The plates were incubated for 1 h at 37° and the wells were washed with buffer B. A solution containing rabbit anti-cCGP (dilution 1:200) and serial dilutions containing either treated newborn plasma or cCGP ($2–5 \times 10^4$ ng/ml) in treated plasma were then added and incubated for 2 h at 37°. After further washing of the plates, 200 μl of the conjugate (dilution 1:1000) was added and incubated for 2 h at 20°. The substrate was then added. The reaction was stopped after 30 min with 50 μl 2 M-$H_2SO_4$. The absorbance at 405 nm was determined.

## RESULTS

### *Measurement of cCGP and hCGP in newborn plasma*

The HPLC elution profile for purified cCGP with the C gradient showed five main peaks at 38, 41, 43, 46 and 47 min (Fig. 1(*a*)). This microheterogeneity was explained by the presence in the cCGP of sugars and phosphoserine. The HPLC elution pattern for control plasma (human plasma from fasting adults) did not present these peaks (see Fig. 1(*a*)). Fig. 2 shows a characteristic elution profile for the plasma from a newborn infant fed on formula ($F_{N1}$); an important peak at 43 min was detected. This peak was also found on the elution profiles for the other $F_N$ and $F_A$ newborn infants but it was smaller.

The HPLC elution profile for purified hCGP with the H gradient shows two peaks at 33 and 34 min (Fig. 1(*b*)). Fig. 3 shows a characteristic HPLC elution profile for plasma from a breast-fed infant; a peak was present at 33 min.

### *N-terminal amino acid sequence analysis of HPLC peaks corresponding to CGP*

Standard plasma (human plasma from fasted adults) containing cCGP or hCGP (1 mg/ml) was chromatographed (Fig. 1(*a* and *b*)). Fractions eluted at 43 min with the C gradient and at 33 min with the H gradient were sequenced. The sequence of the first eleven amino acids

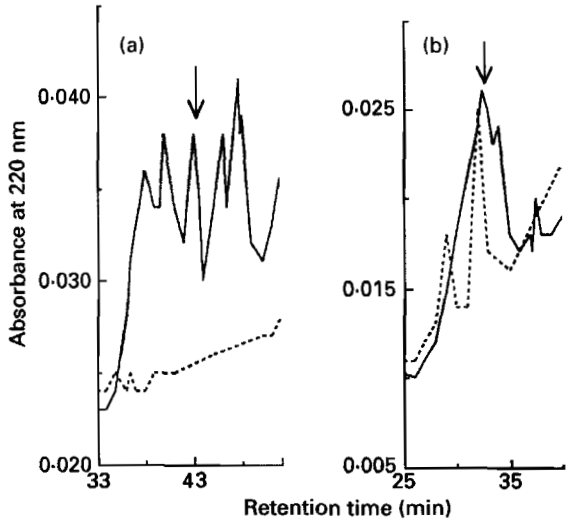


Fig. 1. HPLC elution profiles for (*a*) bovine and (*b*) human κ-caseinoglycopeptides (CGP) with gradients C and H respectively. (——), bovine or human CGP; (----), human plasma from fasted adults; (↓), fraction submitted to Edman degradation. For details of procedures, see p. 584.

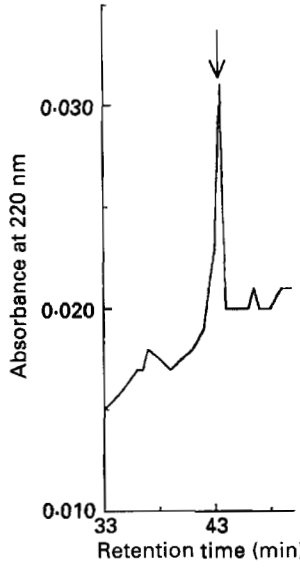


Fig. 2. HPLC elution profile (gradient C; Table 1) for the plasma from a newborn infant fed on cows' -milk-based formula ($F_{N1}$). (↓), Fraction submitted to Edman degradation. For details of procedures, see p. 584.

of cCGP (MAIPPKKNQDK) and of hCGP (IAIPPKKIQDK) and their yields were established (Table 2) and used to calculate the concentrations of cCGP and hCGP in the newborn plasmas. The N-terminal amino acid sequence of cCGP was found in the plasma of three infants fed on formula ($F_{N1}$, $F_{N4}$ and $F_{N5}$) and of hCGP in the plasma of three breast-fed infants ($B_2$, $B_3$ and $B_6$). The estimated concentrations of CGP in newborn plasma

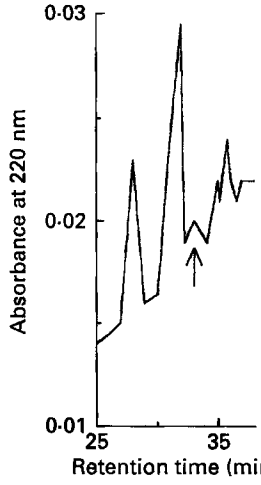


Fig. 3. HPLC elution profile (gradient H; Table 1) for the plasma from a newborn infant fed on human milk ($B_2$). (↑), Fraction submitted to Edman degradation. For details of procedures, see p. 584.

Table 2. *N-terminal amino acid sequence of the HPLC fractions at 43 and 33 min of bovine (c) and human (h) κ-caseinoglycopeptides (CGP) respectively**

(Control values were determined for 100 μg h or cCGP)

| | cCGP | | | hCGP | | |
|---|---|---|---|---|---|---|
| Residue no. | Amino acid | Control (pmol) | Sample ($F_{N1}$) (pmol) | Amino acid | Control (pmol) | Sample ($B_2$) (pmol) |
| 1 | Met | 69 | 1·8 | Ile | 52 | 0·7 |
| 2 | Ala | 59 | 2·5 | Ala | 52 | 1·5 |
| 3 | Ile | 40 | 0·8 | Ile | 61 | 0·3 |
| 4 | Pro | 47 | 1·6 | Pro | 63 | 0·7 |
| 5 | Pro | 45 | 1·7 | Pro | 43 | 0·8 |
| 6 | Lys | 17 | 5·2 | Lys | 28 | 0·2 |
| 7 | Lys | 30 | 1·4 | Lys | 45 | 0·2 |
| 8 | Asn | 32 | 0·9 | Ile | 43 | 0·3 |
| 9 | Gln | 25 | 1·0 | Gln | 31 | 0·5 |
| 10 | Asp | 23 | 0·7 | Asp | 28 | 2·0 |
| 11 | Lys | 16 | 1·2 | Lys | 25 | nd |
| 12 | Thr | 4 | nd | Ile | 27 | 0·3 |

nd, not detected; $F_{N1}$, infant fed on 'Nidal first age' cows'-milk-based formula; $B_2$, breast-fed infant.
* For HPLC profiles, see Fig. 1 (*a* and *b*).
† For details of procedures, see p. 584.

are shown in Table 3. The mean concentrations were 22 and 16 μm/ml for newborn infants fed on formula or breast-fed respectively.

*Measurement of cCGP content in newborn plasma by competitive ELISA*

The anti-cCGP antisera titre was 1/1666. There was no cross-reactivity between cCGP and hCGP. The cCGP content in newborn plasma determined by competitive ELISA is indicated in Table 4. Four cows'-milk-based-formula-fed newborn infants had a cCGP concentration between 4 and 37 μg/ml (mean value 21 μg/ml).

Table 3. *Bovine (c) and human (h) κ-caseinoglycopeptide (CGP) levels in plasma from cows'-milk-based-formula-fed and breast-fed newborn infants determined by N-terminal sequence analysis**

| Newborn infant | Milk source | cCGP (μg/ml) | hCGP (μg/ml) |
|---|---|---|---|
| Formula-fed | | | |
| $F_{N1}$ | Nidal | 30 | |
| $F_{N2}$ | Nidal | nd | |
| $F_{N3}$ | Nidal | nd | |
| $F_{N4}$ | Nidal | 13 | |
| $F_{N5}$ | Nidal | 22 | |
| $F_{A1}$ | Alma | nd | |
| Breast-fed | | | |
| $B_1$ | Human | | nd |
| $B_2$ | Human | | 18 |
| $B_3$ | Human | | 11 |
| $B_4$ | Human | | nd |
| $B_5$ | Human | | nd |
| $B_6$ | Human | | 18 |

nd, not detected.
* For details of procedures, see p. 584.

Table 4. *Bovine κ-caseinoglycopeptide (cCGP) levels in plasma from cows'-milk-based-formula-fed newborn infants determined by competitive ELISA**

(Mean values with their standard errors)

| Newborn infant | cCGP (μg/ml) | |
|---|---|---|
| | Mean | SE |
| $F_{N1}$ | 29 | 0·03 |
| $F_{N2}$ | 37 | 2·96 |
| $F_{N3}$ | 0 | |
| $F_{N4}$ | 4 | 0·01 |
| $F_{N5}$ | 14 | 0·08 |
| $F_{A1}$ | 0 | |

$F_{N1-5}$, 'Nidal first age' formula; $F_{A1}$, 'Alma first age' formula.
* For details of procedures, see pp. 584–585.

## DISCUSSION

We observed previously that hCGP can be detected in rat plasma after ingestion of human casein. The aim of the present study was to detect and quantify an antithrombotic peptide derived from milk proteins (cCGP or hCGP) in newborn plasma. Using protein chemistry techniques we found hCGP in plasma from three of the six breast-fed newborn infants and cCGP in plasma from three of the six formula-fed newborn infants. Immunoenzymic analysis detected cCGP in the blood of four formula-fed newborn infants (one more than could be detected by protein chemistry techniques alone).

HPLC elution profiles for cCGP in newborn plasma do not demonstrate the microheterogeneity observed with the standard cCGP. This suggests the preferential

Table 5. *Protein content of different milks*

| | Total proteins (g/l) | Caseins | | Whey proteins | |
|---|---|---|---|---|---|
| | | g/kg total protein | g/l | g/kg total protein | g/l |
| Human milk | 9–16 | 280 | 2·5–4·5 | 720 | 6·5–11·5 |
| Cows' milk | 35 | 800 | 28 | 200 | 7 |
| Cows'-milk-based formula: | | | | | |
| Nidal* | 20 | 820 | 16·4 | 180 | 3·6 |
| Alma† | 21·5 | 800 | 17·2 | 200 | 4·3 |

* Nestlé, Courbevoie, France.
† Jacquemaire santé, Levallois, France.

passage of a given cCGP through the gastrointestinal barrier. In our study, three formula-fed newborn infants had cCGP showing the same elution profile in their plasma but one probably had another form (not detected by HPLC but giving a positive immunoenzymic reaction).

The mean values for hCGP and cCGP in newborn plasma samples were 16 and 21 $\mu$g/ml respectively; similar values were obtained by both immunoenzymic reactions and protein chemistry techniques. The higher concentration of cCGP may be explained by the greater daily amount of casein ingested by 5-d-old formula-fed newborn infants (about 1 g for breast-fed infants and about 5 g for formula-fed infants; Table 5).

Recently it has been shown that a correlation exists between sugar level and antithrombotic activity; hCGP, which is richer in sugars than cCGP, is more potent (C. Bal dit Sollier, personal communication).

It was important to determine whether the peptides were present at a physiologically active level. It was established that cCGP at 1 mg/kg was antithrombotic in the guinea-pig (C. Bal dit Sollier, personal communication). We found CGP in several newborn plasmas at a concentration between 1 and 3·7 mg/kg, which probably corresponds to an active dose.

During milk digestion other biologically active peptides are liberated. In calves (Yvon & Pélissier, 1987), in rats (Naito & Susuki, 1974) and in pigs (Meisel & Frister, 1989), opioid peptides were found in the gut after milk ingestion. $\beta$-Casomorphin-7 was detected in the intestinal contents of human volunteers who drank cows' milk (Svedberg *et al.* 1985) and permeability of the gut barrier to $\beta$-casomorphins was demonstrated in newborn calves (Umbach *et al.* 1985). We suggest that cCGP or hCGP might be released during $\kappa$-casein digestion and might pass into the blood before enzymic degradation is completed. The newborn gut may be more permeable to the peptide. Previous studies of gastric function in breast-fed infants have also shown that gastric proteolysis is limited by low proteolytic enzyme concentrations and relatively high pH levels (Mason, 1962). Moreover, cCGP inhibited gastric acid secretion, gastrin secretion and gastrointestinal motility in the rat, suggesting that proteolysis may be further depressed by the presence of cCGP in the stomach (Stan & Chernikov, 1982; Stan *et al.* 1983). Our findings appear to confirm these observations.

In conclusion cCGP and hCGP were detected at physiologically active levels in newborn plasma after ingestion of milk. The present study demonstrates an important functional role for small peptides derived from milk proteins. Our findings may have a bearing on the recent epidemiological association between the method of infant feeding, adult serum lipid concentrations and mortality from ischaemic heart disease (Fall *et al.* 1992).

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