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90K (Mac-2 BP) in human milk

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SUMMARY

Human 90K, also known as Mac-2 binding protein (Mac-2 BP), is a secreted glycoprotein which is widely expressed, binds to the human macrophage-associated lectin Mac-2, and may have a role in host defence. We have measured the concentrations of 90K in human breast milk from eight healthy mothers delivering mature healthy infants after full-term pregnancy using a specific immunoenzymatic assay. Maximal 90K concentrations were observed on days 2–3 post-partum, and ranged from 13·4 to 79·2 μg/ml. The concentrations of 90K in the milk had no correlation with those in the maternal blood.

Keywords newborn host defence gastrointestinal infection

INTRODUCTION

The health benefits of human breast milk have been well delineated. Recently, the prophylactic effects of milk in preventing gastrointestinal infections have been described [1–3]. At least in part, this is due to non-immunoglobin glycoproteins present in milk that have binding properties and protect newborns from bacterial or viral infections [4,5]. One of the mechanisms by which these proteins function is by preventing the adherence of the infectious microorganisms to intestinal epithelium. The milk glycoproteins involved in this process have not been characterized. However, a glycoprotein of molecular weight > 400 kD has been described that is able to neutralize respiratory syncytial virus [6]. Similarly, high molecular weight material has been identified in human serum that interferes with the attachment and infectivity of hepatitis A virus to various cell lines [7].

Previously, we have identified a human tumour-derived antigen, designated 90K, in the culture supernatant of human breast cancer cells [8]. A series of analyses have established that 90K is a widely expressed, secreted, 90-kD serum glycoprotein found both in normal individuals and at elevated levels in the serum of cancer patients as well as sufferers from other non-malignant diseases such as HIV [9,10]. Subsequent cloning and sequencing studies have shown that 90K is identical to the human Mac-2 binding protein (Mac-2 BP), a ligand for the lactose/galactose-specific S-type lectin Mac-2 [11]. The biological functions of Mac-2 and its ligand 90K/Mac-2 BP remain unclear. The 32-kD lectin Mac-2, originally identified as a cell-associated macrophage antigen [12], is a bifunctional secreted protein [13], with a lectin domain capable of binding laminin [14] and which, in association with its ligand, 90K/Mac-2 BP, may serve as a bridge between the macrophages and the extracellular matrix, microorganisms, or other cells bearing galactosylated proteins. 90K itself has been shown to be a potent immune stimulator with positive effects on the generation of cytotoxic effector cells (NK/LAK) from human peripheral blood mononuclear cells (PBMC) [11].

Because of the possible involvement of 90K in host defence and the anti-infective and immunomodulatory character of the protection afforded by human milk, we decided to characterize 90K expression in human breast milk. The presence of 90K in human milk has been documented previously [15]. The present study examines the concentrations of 90K in breast milk using a specific immunoenzymatic assay. Values obtained for milk samples were compared with maternal and neonatal blood samples.

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METHODS

Collection of milk and blood

Breast milk samples were obtained with consent form from eight healthy mothers who had delivered mature healthy infants after full-term pregnancy. The study was approved by the Institutional Review Board for Human Research of the University G. D'Annunzio, Chieti. Milk samples were collected on days 1–6 post-partum and 1 month after delivery. The samples were centrifuged at 800 ***g*** for 10 min, cells and lipids were removed and the aqueous layer was collected and stored at -20°C until assayed. Blood samples were obtained from the mothers providing milk specimens. Blood samples were also collected from 12 term healthy infants at 3 days old from the residuum remaining after biochemical analysis for physiological hyperbilirubinaemia. The infants studied were not the infants of mothers who participated in the study for provision of breast milk samples. Blood samples were allowed to clot, and the serum was stored at -20°C until assayed.

ELISA for 90K

A sandwich-type ELISA for the detection of 90K was employed to obtain a quantitative analysis of 90K. This ELISA was developed in our laboratory [9]. Briefly, anti-90K MoAb SP-2 (10 μg/ml) in 0·1 M bicarbonate buffer was added to 96-well flat-bottomed microtitre plates (GIBCO Labs, Grand Island, NY) at 100 μl/well at room temperature for 2 h. Wells were washed 10 times with PBS and then blocked to prevent non-specific adsorption by addition of 200 μl of 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. Wells were flicked empty, and 100 μl/well of test samples diluted 1:200 in PBS were added and allowed to incubate for 1 h at 37°C. The wells were then washed 10 times and incubated with 100 μl horseradish peroxidase (HRP)-labelled SP-2 [9] for 1 h at 37°C. After washing 10 times, wells were incubated with 100 μl chromogen. This consisted of one tablet (5 mg) of *o*-phenylenediamine (OPD; Sigma Chemical Co.) in 12·5 ml of 0·1 M citrate buffer pH 5·0 plus 120 μl of 3% H_2O_2. After development with shaking for 20 min at room temperature, the reaction was stopped by addition of 25 μl of 10% H_2SO_4. Absorbance at 492 nM was read in a Titertek Multiscan reader. A standard titration curve of a known amount of pure 90K antigen was included in each assay, and calculations of the amounts of 90K in test samples were made by linear regression analysis.

This assay is linear in the range of 250 ng/ml to 31 ng/ml. The inter- and intra-assay coefficients of variation were $< 8\%$. The normal serum 90K values in 50 adults range from 2·6 to 11·5 μg/ml (mean 5·7 μg/ml). Pure 90K standard was obtained from the supernatant of cultured NIH 3T3 cells that were transfected with an expression plasmid containing the 90K cDNA [11].

PAGE and immunoblotting of milk 90K

Milk samples were centrifuged at 800 ***g*** for 10 min to remove cells and lipids. Aliquots (10 μl) of the aqueous fraction were heated at 100°C for 3 min in the presence of sample buffer (63 mM Tris–HCl pH 6·8, 4% SDS, 5% mercaptoethanol and 20% glycerol) and electrophoresed for 2 h at 20 mA. A 9% separating gel and a 4% stacking gel were used. After electrophoresis, the gel was electroblotted onto a nylon membrane in blotting buffer at 50 V for 2 h. The membrane was blocked with 5% skimmed dry milk and probed with rabbit anti-90K antiserum for 1 h at room temperature. After washing, the membrane was treated with peroxidase-conjugated goat anti-rabbit IgG, followed by H_2O_2 and OPD [16].

RESULTS

Quantification of 90K in human milk

Milk 90K concentrations were serially measured on days 1–6 post-partum. Maximal 90K concentrations were obtained on days 2–3 post-partum, with a large variation between 13·4 and 79·2 μg/ml, as shown in Fig. 1. Then, milk 90K concentrations declined rapidly to < 10 μg/ml. One month after delivery, milk 90K concentrations were $5{\cdot}3 \pm 4{\cdot}8$ μg/ml.

Molecular form of milk 90K

Immunoblot analysis of 90K in two samples of human milk (Fig. 2) demonstrated the presence of similarly immunoreactive proteins consisting of a major protein band of apparent mol.wt 95 kD and a minor component of $\approx$ 68 kD, probably representing a proteolytic product of 90K as suggested [15]. Thus, the molecular structure of milk 90K was similar to that of serum 90K [16].

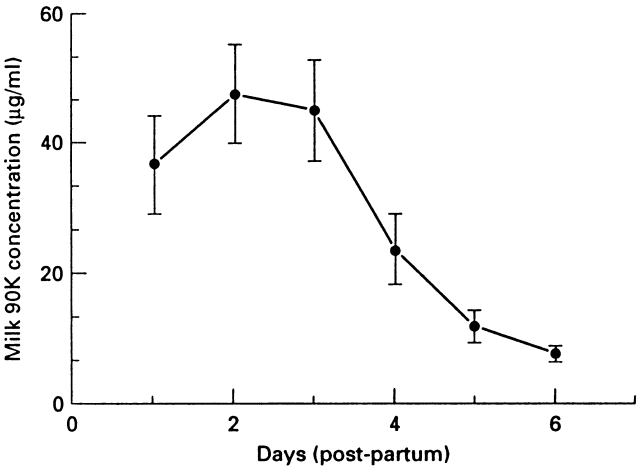

Fig. 1. Serial determination of milk 90K concentration. Each point represents mean values. Vertical bars indicate s.d. of the mean of eight separate 90K measurements.

Simultaneous comparison of milk and serum 90K

To determine whether milk 90K was derived from serum 90K, we examined milk and serum 90K concentrations from the same mothers on day 3 post-partum. As shown in Fig. 3, despite high milk 90K concentrations, serum 90K concentrations were consistently < 10 μg/ml ($P < 0{\cdot}01$ by Pearson correlation analysis). Thus, it is unlikely that milk 90K was derived from serum. Finally, the 90K concentration in day 3 milk was $\approx$ 5-fold higher than in neonatal blood and $\approx$ 10 times higher than in adult blood (Fig. 4)

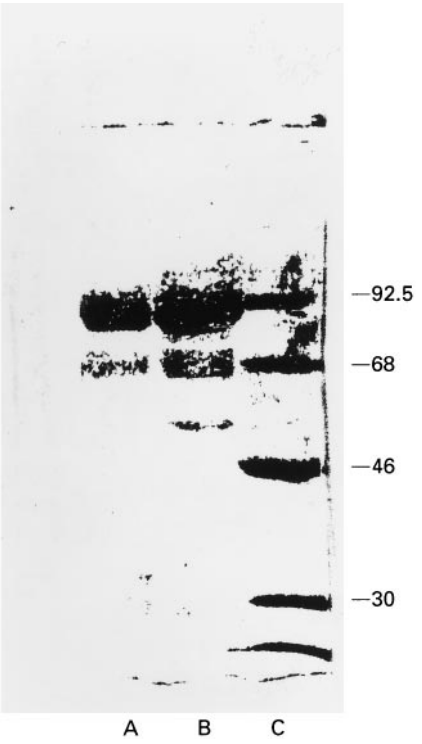

Fig. 2. Western blot analysis of 90K in human milk. Aliquots (10 μl) of milk were analysed on a 9% gel and proteins blotted onto a nylon membrane. Lane A, milk sample containing 31 μg 90K/ml; lane B, milk sample containing 49 μg 90K/ml. Migration positions of molecular weight markers (lane C, kD) are shown.

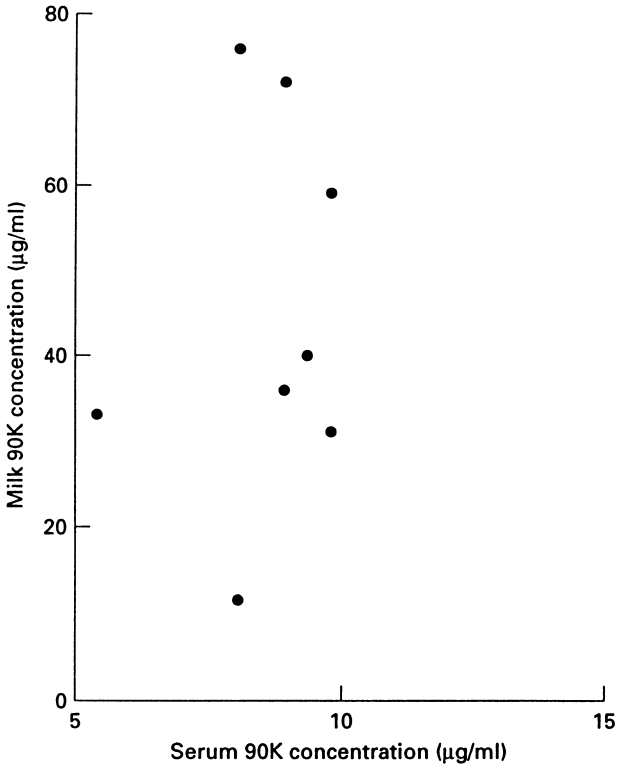

Fig. 3. Simultaneous determination of milk and serum 90K concentrations.

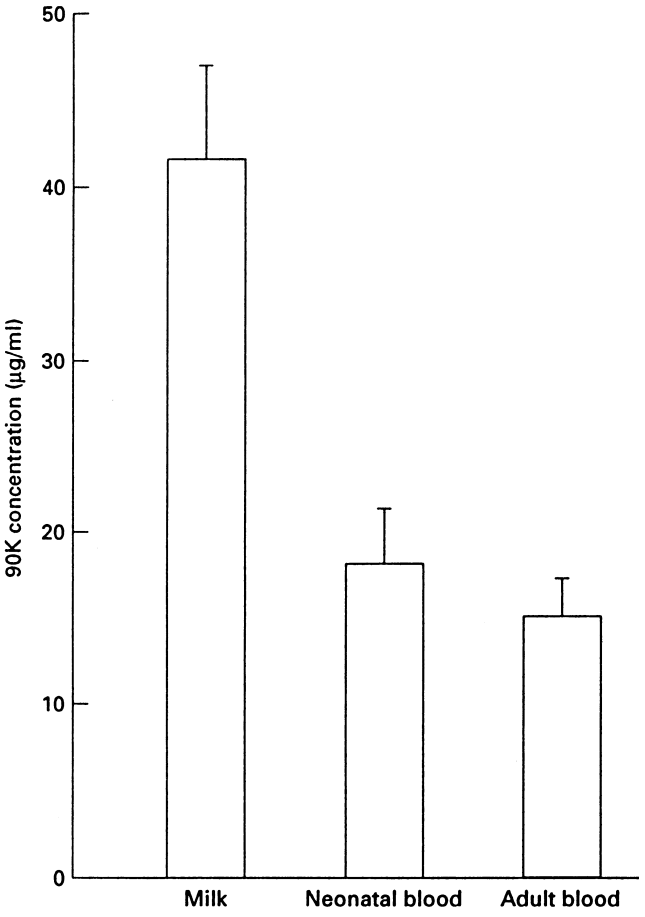

Fig. 4. 90K concentrations in milk, and in neonatal and adult blood. Bars represent the mean ± s.e.m.

DISCUSSION

Human breast milk contains a variety of cellular and soluble components that may protect breast-fed neonate from infections [17,18]. In the present study we have found that human milk contains a large amount of 90K. The concentrations of the protein were highest at days 2–3 post-partum. Based upon the average amount of milk secreted during the first days of lactation [19] and the mean concentration of 90K in milk (42 μg/ml) (120 ml/day $\times$ 42 μg/ml), about 5 mg/day of 90K are secreted in milk. The levels of 90K in breast milk are high compared with serum from healthy neonates. Thus, the milk may provide 90K before its full production is reached by the recipient.

Evidence for a role of 90K and its ligand Mac-2 in host defence functions has been provided. 90K belongs to an ancient protein superfamily which is defined by a scavenger receptor cystein-rich (SRCR) domain [11]. It is particularly noteworthy that all the previously characterized SRCR domains occur in proteins involved or thought to participate in critical functions of the cellular host defence system, and thus they resemble the immunoglobulin superfamily of the cystein-containing protein domains (reviewed in [20]). Histochemically, Mac-2 has been located intracellularly in many members of the macrophage lineage, including tissue macrophages, certain dendritic cells, alveolar and Kuppfer cells [21,22]. Additionally, Mac-2 was shown to be present in some epithelia including kidney and intestine [23]. In these tissues, Mac-2 expression occurs predominantly at apical surface, and interestingly in intestine, expression is prominent in the tips of villi, where it may serve as a target for colonization by pathogens. Finally, specific 90K mRNA expression was shown to occur prominently in tissues containing cavity lining secretory epithelia (stomach, duodenum, colon) [11], where it may function as an immune-stimulatory and anti-infective agent by interfering with the adherence of pathogens to the surface of Mac-2 expressing cells.

In conclusion, we suggest that milk 90K may have a beneficial effect on the neonate gastrointestinal tract by affording protection against infections. Once this role of 90K is firmly established, it will be possible to monitor 90K concentration in human milk to determine if an infant is receiving the appropriate amount of this protein for maximum health benefit.

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