

Does Calprotectin Represent a Regulatory Factor in Host Defense or a Drug Target in Inflammatory Disease?

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Abstract: Calprotectin, a protein composed by two subunits of 8 and 14 kD respectively, is released by neutrophils in the biological fluids under inflammatory states. For instance, detection of calprotectin in faeces represents a diagnostic tool in the case of inflammatory bowel disease. Quite interestingly, calprotectin is increased in the stool of healthy newborns from day three up to day thirty and, physiologically, this increase may be interpreted as a defense mechanism against yeast and fungi. Therapeutic attempts at inhibiting the deleterious effect of calprotectin have been experimentally made by using lycoricidinol. This natural compound is able to hamper the calprotectin-induced apoptosis on the one hand. On the other hand, the same compound plays a prophylactic role in the course of experimental arthritis in rats.

Key Words: Calprotectin, inflammatory bowel disease, newborns, lycoricidinol.

INTRODUCTION

Calprotectin (MRP8/14, S100A8/S100A9, 27E10 antigen) is a calcium- and zinc-binding protein complex composed of 8 and 14 kD subunits [1]. These 8 and 14 kD peptides belong to the S100 protein family which is a subfamily of proteins with Ca⁺⁺-binding motif, EF-hand in each molecule. These subunits have been also termed L1 light and heavy chains [2], p8 and p14 [3], migration inhibitory factor-related protein (MRP)8 and MRP14 [4] and calgranulin A and B [5]. The gene cluster of the S100 proteins is located on human chromosome 1q21, and the nomenclature of proteins was established according to the organization of these S-100 genes [6]. Therefore, the terms S100A8 and S100A9 are now common, although the names MRP8 and 14, or calgranulin A and B, are still often used. Calprotectin is present in the cytoplasm of neutrophils as well expressed on the membrane of monocytes.

Upon neutrophil activation or endothelial adhesion of monocytes, calprotectin is released and may be detected in serum, body fluids and faeces as a potentially useful clinical inflammatory marker. A high level of calprotectin reportedly exists in extracellular fluid during various inflammatory conditions, such as rheumatoid arthritis [7], cystic fibrosis [8,9] and active multiple sclerosis [10]. The soluble form of calprotectin provides both bacteriostatic and cytokine-like effects in the local environment [11]. When calprotectin metabolism is affected on a systemic level, the zinc-binding properties of protein may induce severe dysregulation of zinc homeostasis with severe clinical symptoms. The distribution of membrane form of calprotectin is restricted to monocytes and immature macrophages and the presence of calprotectin-positive infiltrating cells reflects the influx of mononuclear

phagocytes to the site of inflammation. The intracellular distribution of calprotectin, on the other hand, varies with the activation state of macrophages. Normal macrophages contain the protein complex in the cytosolic fraction, but, once stimulated, this complex translocates to the cell membrane, thus localizing with proteins of the cytoskeleton [10]. This implies that calprotectin may be related to cell movement, phagocytosis or signal transduction.

Although calprotectin has been used as a specific marker for neutrophils and macrophages, it has been also detected in other cell types. As an example, it has been detected in keratinocytes in inflammatory dermatoses [12], and squamous cell carcinoma. Also, the 14 kD subunit of calprotectin is expressed in a subset of microglia in brain tissue with Alzheimer's disease [13]. Since the expression of calprotectin in these cell types seemed to be up-regulated by the inflamed state of the tissue, the functional relevance of the factor to each inflammatory process was suggested [5,12,13].

In synthesis, Calprotectin expression and release seem to be of particular importance in immune and immunopathological reactions. However, the exact biological role(s) of the factor is now under investigation.

Yui *et al.* [14] recently observed that neutrophils contain a factor that shows growth-inhibitory and apoptosis-inducing activities against various cell types, even including tumor cells and normal fibroblasts, and identified that factor as calprotectin. Calprotectin induces apoptosis through a dual mechanism. One is the zinc exclusion from the target cells, and the other is the binding of the factor to target cell surface, possibly in a ligand-receptor fashion. Determination of the mode of interaction between calprotectin and cell surface receptor for tumor cells is an important hurdle in solving the apoptosis-inducing mechanism of the factor, in addition to identifying the mechanism of zinc inhibition.

The overall findings suggest that calprotectin exerts a regulatory activity in inflammatory processes through its

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effect on the survival or growth states of cells participating in the inflammatory reaction. It is likely that calprotectin, at a higher concentration, might have a deleterious effect on fibroblasts, also influencing the recovery of inflammatory tissue.

FAECAL CALPROTECTIN AND DIAGNOSIS OF GASTROINTESTINAL DISEASE

When calprotectin is bound to calcium, it is remarkably stable. It is, for instance, stable in faeces stored for 7 days at room temperature [15]. A faecal calprotectin enzyme-linked immunosorbent assay (ELISA) has been available since 1994 [16].

Clinically, chronic inflammatory bowel disease (IBD) is characterized by periods of well being interspersed by exacerbations of disease activity. Differentiation between IBD and less severe disorders such as irritable bowel syndrome (IBS) requires invasive and expensive diagnostic procedures. Diagnostic differentiation between active disease, symptoms due to residual constriction of the fibrotic lumen and functional symptoms are a well-known problem. In this context, there are not yet any laboratory parameters with sufficient discrimination in terms of sensitivity and specificity. Colonoscopy and histopathological examination remain the gold standards, and in Crohn's disease this may be due to the variable localization of the inflammatory process. At the same time, abdominal scintigraphic procedures, although informative, are complex and expensive. The recent assessment of faecal calprotectin offers an attractive alternative as an index of intestinal inflammation [17]. Assessment of faecal calprotectin concentration can be used as a screening test--an 'Erythrocytes Sedimentation Rate of the gut'--to select patients for further examination. The test can be performed on little amount (0.5 g) of random stool samples that can be sent to the laboratory by ordinary mail since the protein is remarkably stable in stools. Single stool assay of neutrophil-specific proteins (calprotectin, lactoferrin) generates the same quantitative data on intestinal inflammation as the 4-day faecal excretion of indium-111-labeled white cells, suggesting that faecal calprotectin reflects the granulocyte migration through the gut wall [18].

Berstad *et al.* [19] found a significant correlation between calprotectin concentration in gut lavage fluid and intestinal permeability, suggesting that increased intestinal permeability in IBD may be a consequence of increased transepithelial migration of neutrophils. Elevated levels of faecal calprotectin have been demonstrated in patients with Non Steroid Antiinflammatory Drug-induced enteropathy [20] and have been utilized in the diagnosis of colorectal cancer [21,22]. Faecal calprotectin is increased in over 95% of patients with IBD and correlates with clinical disease activity [23,24]. Its determination reliably differentiates between patients with IBD and IBS. More importantly, at a given faecal calprotectin concentration in patients with quiescent IBD, the test has a specificity and sensitivity in excess of 85% in predicting clinical relapse of disease [25,26] This suggests that relapse of IBD is closely related to the degree of intestinal inflammation [27,28], and suggests that targeted treatment at an asymptomatic stage of the disease may be indicated

Calprotectin seems to cause tissue destruction when it is present in an excess amount in local inflammatory tissue for a long period. The protein complex is an injurious factor of neutrophils. If so, is there any beneficial aspect of calprotectin activity, or is it merely a toxic substance that nature capriciously endowed to the body? At least in an abscess condition, it seems probable that growth inhibition or apoptosis of the surrounding fibroblasts by calprotectin has a positive effect in curing of the abscess. Infact, destruction of fibroblasts constituting connective tissue may hasten the excretion of the abscess contents which includes dead and viable inflammatory cells (the source of calprotectin) and microorganisms.

FAECAL CALPROTECTIN IN PEDIATRIC PATIENTS AND IN NEWBORNS

A recent study demonstrates that faecal calprotectin is a sensitive but not disease specific marker useful in easily detecting inflammation throughout the whole gastrointestinal tract in children [29]. The authors showed an increase in faecal calprotectin values in those paediatric gastrointestinal diseases characterized by mucosal inflammation, while children affected by functional disorders or by non-inflammatory diseases had normal values. In healthy children from 2 to 18 years calprotectin reference values are higher than in adults [30].

Faecal calprotectin could be an interesting marker to evaluate intestinal status in newborns, particularly in those at risk of necrotizing enterocolitis, such as preterms and newborns with intrauterine growth retardation or perinatal asphyxia. Correl *et al.* [31] showed that preterm newborns with clinical features of necrotizing enterocolitis had raised faecal calprotectin concentrations at the time of diagnosis compared with matched controls [288.4 mg/L (SD 49.1) and 98.0 mg/L (60.6), respectively; $p=0.0006$]. Calprotectin is already present in the first passed meconium [32], with higher levels in preterms and low birthweight neonates, as well as in neonates with some degree of perinatal asphyxia, as indicated by the negative correlation with 5'-Apgar score. These findings are probably secondary to either the immaturity of the intestinal mucosa or its hypoxic-ischaemic damage.

A recent study [33] has reported higher concentrations of faecal calprotectin during the first few days of life, without influence on the mode of feeding. Some authors demonstrated that, when the stools are collected from a paper diaper, water absorption into the diaper may increase the concentration of calprotectin by up to 30% [34]. However, this finding cannot justify per se the higher calprotectin levels in newborns. On the other hand, lower levels of calprotectin in human milk are reported [34].

CALPROTECTIN VALUES IN HEALTHY FULL TERM NEWBORNS DURING THE FIRST MONTH OF LIFE

Even if recent studies demonstrated an age-dependent variation in faecal calprotectin in children [35], there are no data about calprotectin values during the first month of life. In this framework, aim of our study was to measure faecal calprotectin concentrations in healthy newborns, in different days during the first month of life, and to define values ac-

ording to the gender, the mode of delivery, the type of feeding (standard formula vs mother milk).

A perspective study was carried out in 71 full term healthy newborns admitted to the Neonatology-Neonatal Intensive Care Unit of the Department of Biomedicine of Evolutive Age of the University of Bari (Bari, Italy), from March to September 2005. The subjects included 38 males and 33 females, 31 born by caesarean section and 40 by the vaginal route with a mean gestational age of 39,21 weeks (range 37-41) and a mean birth weight of 3306,19 gr (range 2650-4000). Fifty newborns received mother milk and 21 a standard formula. Four stool samples were taken from each newborn on day 3, 7, 12 and 30 and were stored in plastic containers, and, then, frozen at -20° until use.

For calprotectin assay, 40-120 mg feces were collected from the diaper with a disposable loop and placed in a sterile 14-ml screw-cap tube and then mixed with an extraction solution containing urea and citrate in a weight: volume ratio of 1:50. After 30 sec. agitation on a mixer and homogenization for 20 min. at 1400 rpm, 1 ml of homogenate was transferred to an eppendorf tube and centrifuged for 20 min at 10.000 x g. the supernatant (0.5 ml) was, then, collected, and immediately analyzed with a calprotectin ELISA, with standards and controls included, performed according to the manufacturer's instructions (Calprest, Eurospital, Trieste Italy). The reported analytic sensitivity of the assay was 6,25 ng/ml, corresponding to 15.6 mg calprotectin kg⁻¹ faeces.

Statistical analysis. Data were analyzed using the SPSS statistical software package. Difference between means were evaluated using unpaired t-test. For repeated measures ANOVA was used to evaluate calprotectin increase. P-values of less than 0.05 indicated statistically significant differences.

Two hundred and seventyfour samples were collected and faecal calprotectin analyzed.

Calprotectin values (means, medians, range, SD, CI) are shown in Table 1 and in Fig. (1). From day 3 to day 7 of life calprotectin levels increased in a statistically significant way (p<0.0001), and with a moderate increase up to the day 30. No differences were found in relation to gender, the mode of delivery and type of feeding (data not shown).

DISCUSSION

According to current literature faecal calprotectin concentrations are higher in newborns compared with concentrations in adults and healthy children. As far as cellular source of calprotectin is concerned, Yui *et al.* [36] studied the

course of intracellular and extracellular calprotectin expression in inflammatory cells using a rat peritonitis model. In response to an intraperitoneal injection of heat-killed *Enterococcus faecalis*, into Wistar rats, neutrophils immigrated into the peritoneal cavity after 6 hours, with a peak around day 2. In a later phase, macrophage accumulation was observed. Although calprotectin was reportedly expressed in both types of exudate cells [37], the amount of the factor in the ascitic fluid was not related to the appearance of macrophages, rather it paralleled the degree of neutrophil infiltration. Therefore, neutrophils, rather than macrophages, were the most important source of extracellular calprotectin in this experimental model. Likely, the factor can be released from necrotic neutrophils, although an active secretory mechanism cannot be ruled out [38,39]. Under physiological circumstances, epithelia contain intraepithelial lymphocytes only, as seen on small intestinal biopsies from infants [40, 41]. Therefore, increased transepithelial migration of either neutrophils or newly recruited macrophages could account for the high level of calprotectin.

Starting at birth, humans and other mammals are colonized with diverse societies of bacteria that cover the surfaces of the gastrointestinal tract. The microbes are in continuous and intimate contact with host tissues. The intestinal epithelium, in addition to mount a physical barrier to microbial penetration, plays a protective role by producing and secreting large quantities of antimicrobial peptides. This keeps commensal bacteria from penetrating host tissues and causing serious problems as inflammation and sepsis [42]. Of note, small-intestinal Paneth cells are key effectors of this type of innate host defense [43,44]. Otherwise, during the first weeks of life potent chemotactic agents produced by the intestinal flora, such as formyl-methionyl-leucil-phenylalanine (FLMP), may stimulate the transepithelial migration of granulocytes [45], generating a higher intraluminal concentration of calprotectin as a defense against invading or attaching pathogen agents. Calprotectin, in fact, suppresses the growth of yeast and fungi [46,47]. In this respect, it was reported that calprotectin cooperates with neutrophils and/or their product, lactoferrin, to inhibit the growth of *Candida albicans*. Thus, it can be concluded that calprotectin, in concert with other factors, contributes to the host defense mechanism against fungal infection.

Calprotectin inhibits the immunoglobulin synthesis of B lymphocytes [48], thus suggesting that it might affect not only inflammatory processes, but immune responsiveness. In any event, evidence has recently accumulated that in addition to its cytotoxic activities, calprotectin possesses a cytokine-

Table 1. Faecal Calprotectin Levels at Different Days of Life in 71 Newborns

Days	Min	Max	Mean	95 % C.I.	Median	SD.
3	28	350	188.34	171.97-204.73	195	68.18
7	95	405	237.67	218.18-257.15	245	81.10
12	80	425	246.08	225.16-267.01	250	88.42
30	100	425	259.61	241.63-277.59	255	75.43

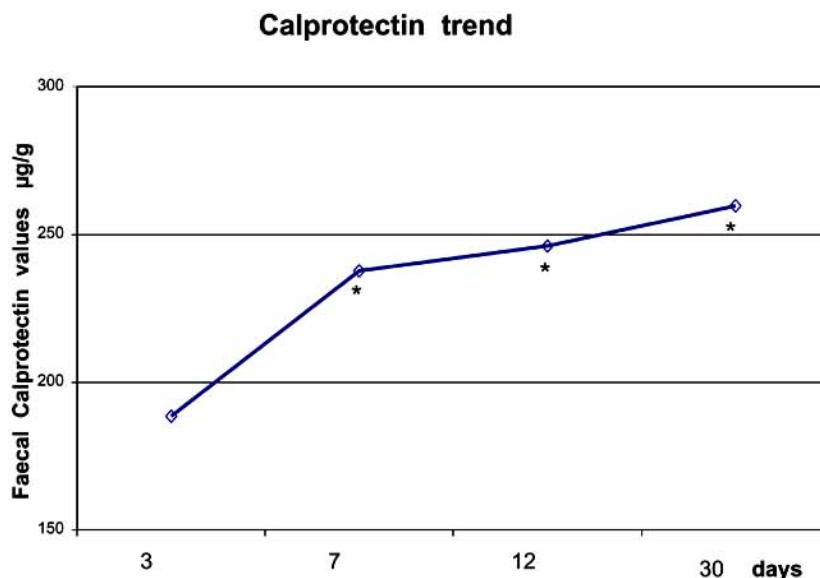


Fig. (1). Calprotectin values in 71 newborns at different days of life.
* $P < 0.0001$. All the values are statistically significant vs day 3.

like activity. It is, therefore, likely to postulate that the protein complex or the individual subunits are not only a useful marker of inflammatory states, but also an important mediator with multiple regulatory functions in inflammatory reactions.

As the knowledge regarding calprotectin accumulates, the protein complex is strongly expected to gain a position as a mediator in inflammation but also perhaps to exert a positive effect on the host defense in physiological conditions, as in healthy newborns.

Drug targeting calprotectin might be an important goal in regulating inflammatory reactions, thus opening new avenues to novel antiinflammatory compounds. Since calprotectin might cause tissue destruction through its growth inhibitory or cytotoxic effects, under severe inflammatory conditions, Yui *et al.* [36] searched for drugs able to suppress the cytotoxic effect of this factor. Using a tumor cell line as a target, they screened plant products that have been used in Chinese medicine for their antiinflammatory activity. They found that the extract of *Crinum asiaticum* exhibited a strong inhibitory activity against calprotectin induced cell death among hot water extracts of 59 plant species. Through purification studies, the molecule responsible for the inhibition was identified as the *Amaryllidaceae* alkaloid, lycorine. Lycorine inhibited not only calprotectin-induced apoptosis, but also its growth-inhibitory effects at a half effective concentration of 0.1–0.5/ µg/ml. Although lycorine displays inhibitory activity against protein synthesis on ribosomal translation, 81–83-, the inhibition occurred at a more than 5-fold higher concentration than that required by the inhibition of calprotectin activity. Since lycorine seems to act in the early induction phase of calprotectin-mediated apoptosis, this may represent a tool for analyzing common pathway in cell death signal transduction.

The group of *Amaryllidaceae* alkaloids contains many biologically active compounds, which include cytotoxic and

antiviral substances [49]. Among these, lycoricidinol (narciclasine) displays a very potent inhibitory activity on the protein synthesis of mammalian cells [50]. The lycoricidinol inhibited calprotectin-induced cytotoxicity at a more than 10-fold lower concentration than lycorine, causing 50% inhibition at 10ng/ml. Moreover, other authors [51] examined the prophylactic effect of lycoricidinol on the rat adjuvant arthritis model, since calprotectin has been reported to be increased in the local inflammatory sites of rheumatoid arthritis patients [52,53,54]. It was found [51] that lycoricidinol exhibited a significant prophylactic effect on the arthritis model, as indicated by suppression of the degree of swelling in adjuvant-treated as well as untreated feet. However, it is, of course, necessary to examine whether calprotectin inhibition is the true mechanism of the prophylactic effect.

In conclusion, these results suggest that lycoricidinol might be a candidate as a drug with suppressive activity toward calprotectin-induced inflammation [51].

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