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REVIEW

Procalcitonin in sepsis and systemic inflammation: a harmful biomarker and a therapeutic target

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The worldwide yearly mortality from sepsis is substantial, greater than that of cancer of the lung and breast combined. Moreover, its incidence is increasing, and its response to therapy has not appreciably improved. In this condition, the secretion of procalcitonin (ProCT), the prohormone of calcitonin, is augmented greatly, attaining levels up to thousands of fold of normal. This hypersecretion emanates from multiple tissues throughout the body that are not traditionally viewed as being endocrine. The serum values of ProCT correlate with the severity of sepsis; they recede with its improvement and worsen with exacerbation. Accordingly, as highlighted in this review, serum ProCT has become useful as a biomarker to assist in the diagnosis of sepsis, as well as related infectious or inflammatory conditions. It is also a useful monitor of the clinical course and prognosis, and sensitive and specific assays have been developed for its measurement. Moreover, it has been demonstrated that the administration of ProCT to septic animals greatly increases mortality, and several toxic effects of ProCT have been elucidated by *in vitro* experimental studies. Antibodies have been developed that neutralize the harmful effects of ProCT, and their use markedly decreases the symptomatology and mortality of animals that harbour a highly virulent sepsis analogous to that occurring in humans. This therapy is facilitated by the long duration of serum ProCT elevation, which allows for a broad window of therapeutic opportunity. An experimental groundwork has been established that suggests a potential applicability of such therapy in septic humans.
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Introduction

Millions of patients in the world are increasingly exposed to sepsis each year, and the rate of sepsis mortality remains high (Angus *et al.*, 2001; Harrison *et al.*, 2006). Although sepsis is a dynamic and complex syndrome, certain serum markers have been implicated as playing a central, and potentially harmful, mediator role in this acute and devastating illness, for example, endotoxin, cytokines, chemokines, prostaglandins, oxygen free radicals, etc. Over several decades, various studies utilizing strategies, such as antibodies, soluble receptors or receptor antagonists, were initiated with much enthusiasm, only to be terminated with high cost and much chagrin due to lack of therapeutic impact. Indeed, based on these failures, it has became commonplace to conclude that such therapy is doomed to failure.

The present review demonstrates that many studies now indicate that the prohormone, procalcitonin (ProCT), is an excellent marker for sepsis and its related conditions, and that the immunoneutralization of this prohormone offers considerable therapeutic promise.

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Sepsis and related conditions

The clinical term *sepsis* is characterized by a marked attack upon the host by pro-inflammatory cytokines that has been precipitated by an infection. Symptomatically, this illness often is manifested by two or more of the following: fever or hypothermia, tachypnea, tachycardia, leukocytosis or leukopenia. Not uncommonly, sepsis leads to one or more severe complications: for example, hypotension, cardiac failure, coma, renal failure, intravascular coagulation. This phenomenon is termed *multiple organ dysfunction* (Fry, 2000; Jean-Baptiste, 2007). Frequently, the disease leads to death. The mortality is greatest in infancy, the elderly, patients with other illnesses and the immunocompromised. When the offending microorganism is identified, bacteria are most often found to be the culprits. However, an identical condition is induced by the malarial parasite, and, rarely, by a fungal or viral infection.

Initially, a consensus meeting recommended that the term sepsis should be reserved for a patient with an infection

(ACCP-SCCM Consensus Conference, 1992). However, it has been shown that microbiological cultures in patients strongly suspected of having sepsis are positive in only about 50% of cases, partially due to technical problems of the culture and the timing of specimen collection. Accordingly, it was concluded that the infection may only be strongly suspected, without being microbiologically confirmed (Levy *et al.*, 2003). While some authors still insist on sepsis being characterized by proven infection, many others make the clinical diagnosis if infection is presumed or suspected even though not proven (Seam and Suffredini, 2007; Chen *et al.*, 2009). Consequently, the terms *culture-positive* and *culture-negative* sepsis have been employed; the symptomatology and mortality of these two classifications have been reported to be similar (Rangel-Frausto, 1999). In sepsis, when cultures are positive, Gram-negative bacteria are moderately more common than Gram positive. However, the prevalence of Gram-positive bacterial pathogens is rapidly increasing (Opal and Cohen, 1999).

The systemic inflammatory response syndrome (SIRS) is the term given to patients who have two or more of the clinical symptoms noted in sepsis: fever or hypothermia, tachypnea, tachycardia and leukocytosis or leukopenia, although infection is not deemed to be present. As in sepsis, multiple organ dysfunction and death may also occur. Clearly, in view of the problems with bacterial culture mentioned earlier, the distinction between SIRS and sepsis is blurred. While in clinical practice, fever, hypothermia, tachypnea, tachycardia and/or leukocytosis or leukopenia can be non-specific findings, some authors believe that there is a hierarchical continuum towards sepsis (Rangel-Frausto, 1999; Brun-Buisson, 2000).

Conditions usually given the appellation SIRS include trauma, extensive surgery, heat stroke, pancreatitis, respiratory distress syndrome and burns. Importantly, as in sepsis, these conditions are frequently characterized by a hypersecretion of pro-inflammatory cytokines (Bone, 1996). It has been demonstrated that in these illnesses, there may be translocation across the gut wall, or across the respiratory or urogenital epithelial barriers of bacteria or of bacterial constituents, which are known to be potent stimuli to the secretion of pro-inflammatory cytokines (Berg, 1992; Ryan *et al.*, 1992; Guyer *et al.*, 2000; Li *et al.*, 2001; Ammori *et al.*, 2003a; Wang *et al.*, 2003; Shibata *et al.*, 2005; Duff *et al.*, 2006; Pezzicoli *et al.*, 2008). The same syndrome may also arise from non-bacterial sources, originating from the release of factors from dying cells, that is, damage-associated molecular patterns (Cinel and Opal, 2009).

Lymphocytes and their subsets (T cells, B cells and natural killer cells, as well as monocytes) are a basic part of the immune system, and these cells, along with neutrophils, play a major role in defending the host from infection when they are functioning appropriately (Majlessi *et al.*, 2008; Zucchini *et al.*, 2008; Ermert *et al.*, 2009). These cells, as well as many others, secrete cytokines, which act as signalling peptides permitting different cells and tissues to intercommunicate and interact with one another. In sepsis, an expanding number of cytokines have been found to be involved in the pathophysiology of the disease (Bozza *et al.*, 2007). For example, TNFα is an intrinsic part of the pro-inflammatory process; it influences immune cells, and can cause cell death. IL-1β, among other effects, induces fever, enables leukocytes to cross the capillary endothelium and increases sensitivity to pain; it is cytotoxic to various cell types and activates the caspase cascade of apoptosis. IL-6 is a multifunctional cytokine that is largely causative of the acute-phase response that follows injury and infection. Some actions of these and other pro-inflammatory cytokines are in part beneficial, but when they are over-expressed, they are counterproductive and lead to worsening of the illness or death (Slifka and Whitton, 2000; Bozza *et al.*, 2007; Cinel and Opal, 2009).

ProCT

ProCT is the precursor for the hormone calcitonin (CT) (Roos *et al.*, 1974; Jullienne *et al.*, 1980). CT, which is found in the thyroid C cells and the pulmonary endocrine cells, has a metabolic role in calcium homeostasis (Hirsch *et al.*, 1963; Zaidi *et al.*, 1992). The amino acid sequence of ProCT is comprised of 116 amino acids (LeMoullec *et al.*, 1984). It is composed of a centrally placed 33-amino acid immature CT that is not amidated, a 57-amino acid aminoterminus (NProCT) and a 21-amino acid CT carboxypeptide 1 (CCP1) at the carboxyl terminus (Figures 1 and 2). All of these component peptides, including ProCT, have been found to circulate at very low concentrations in normal serum, presumably produced by the neuroendocrine cells in the thyroid gland and in the lungs (Snider *et al.*, 1997). However, all tissues throughout the body have the potential to elaborate ProCT (see below).

Hyperprocalcitonaemia in sepsis and related conditons

In sepsis, systemic infection and severe inflammation, the serum levels of ProCT usually increase markedly, attaining values of tens, to hundreds, to thousands-fold that of normal levels (Assicot *et al.*, 1993; Whang *et al.*, 1998; Müller *et al.*, 2000). The same phenomenon has been found to occur in

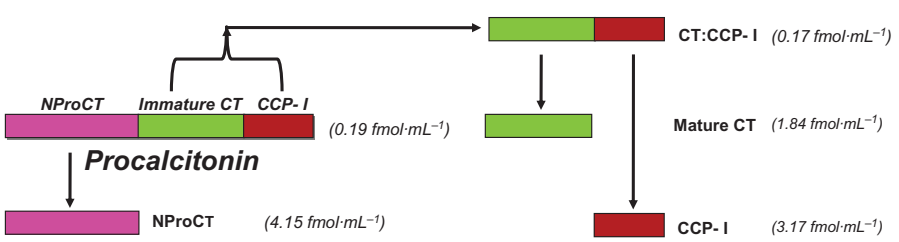

Figure 1 Procalcitonin and its constituent peptides in normal serum, all of which are found at the indicated low concentrations in the blood of normal humans (Snider *et al.*, 1997).

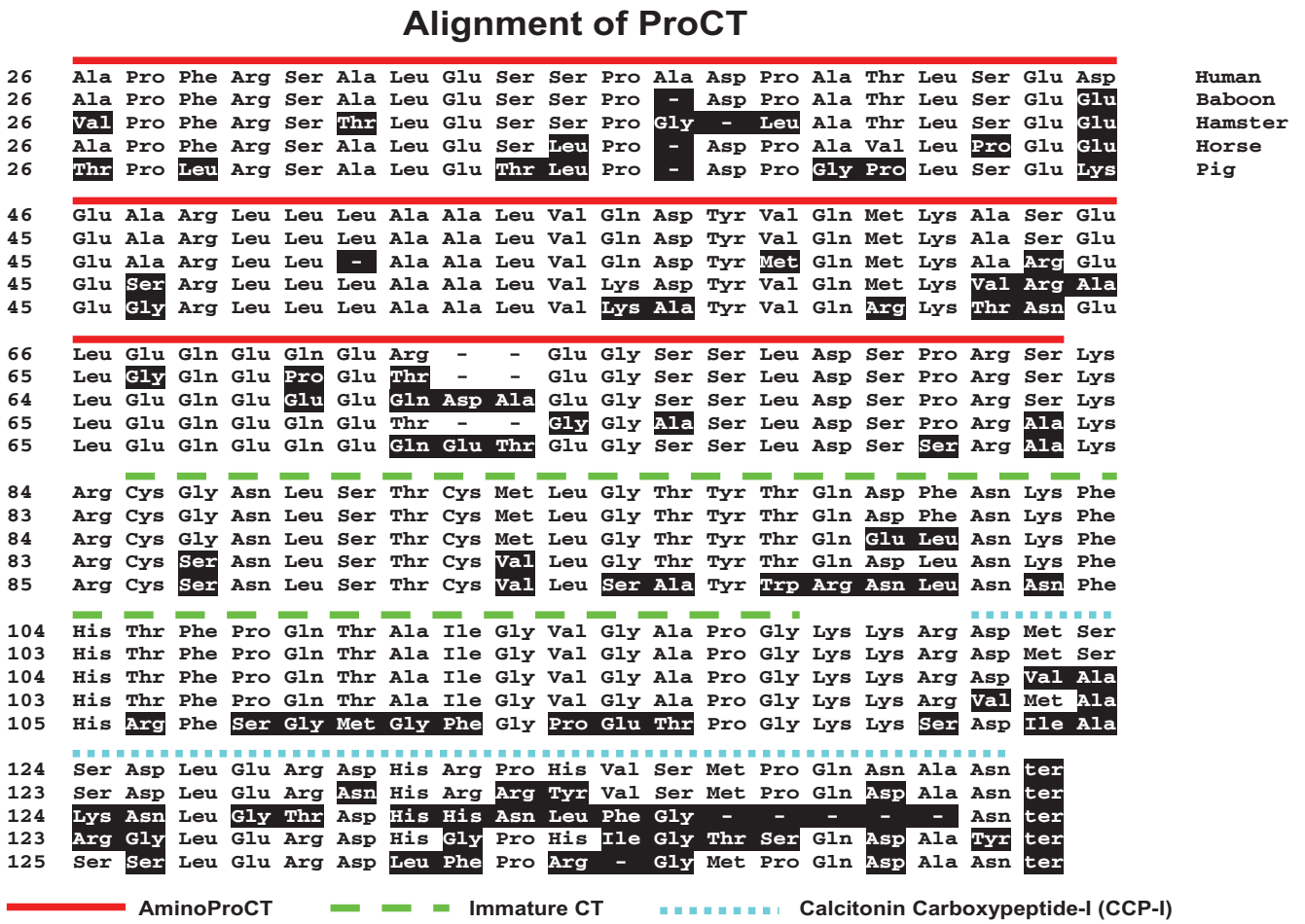

Figure 2 Comparative amino acid sequences of procalcitonin (ProCT) of different species. The human prohormone is derived from the CALC-I gene, via an alternative mRNA splicing that gives rise to the inclusion or exclusion of exons (Amara *et al.*, 1982; Becker *et al.*, 2002). The gene produces three different messenger RNAs. Two of these, after translation, eventuate into calcitonin precursor molecules. The first 25 amino acids that reside at the amino terminus of human pre-procalcitonin comprise the hydrophobic signal peptide that directs the prohormone through the endoplasmic reticulum. The following 57 amino acids comprise N-procalcitonin (NProCT), the next 33 amino acid residues is the immature calcitonin (the distal glycine residue is deleted if the calcitonin becomes amidated and is freed from the prohormone). The lysine–arginine at the terminus of NProCT and the lysine–lysine–arginine amino acids at the distal end of calcitonin are basic amino acid sites of cleavage. The 21 amino acid calcitonin carboxypeptide (CCP-I) is at the most distal end of the prohormone. (CCP-II, found in small concentrations in thyroid, pituitary and nervous tissue, differs by eight amino acids). All of the ProCT peptides are freed during the processing of the prohormone. The structure of human ProCT was reported by Dr Le Moullec *et al.*, Paris, France (LeMoullec *et al* 1984); baboon ProCT was determined by Dr Andrew Russo *et al.*, Iowa City, IA, USA; horse ProCT (Toribio *et al.*, 2003); hamster and porcine ProCT determined by Dr Beat Muller *et al.*, Aarau, Switzerland.

several species of animals, that is, hamster, rat, pig and baboon (Nylén *et al.*, 1998; Redl *et al.*, 2000; Wagner *et al.*, 2002). Other than in some neuroendocrine tumours, clinical studies in humans reveal that the highest serum ProCT values occur in patients with sepsis. They also are increased in pneumonia (Nylén *et al.*, 1996); acute inhalational injury (Nylén *et al.*, 1992); and other severe infections and inflammations such as pancreatitis (Ammori *et al.*, 2003b), appendicitis (Kafetzis *et al.*, 2005), burns (Von Heimburg *et al.*, 1998), heat stroke (Nylén *et al.*, 1997), multitrauma (Maier *et al.*, 2009) and extensive surgery (Meisner *et al.*, 1998). While specific assay of serum ProCT in the healthy subject is less than 10 $pg \cdot mL^{-1}$ (Snider *et al.*, 1997), it is not uncommon for levels to exceed 100 000 $pg \cdot mL^{-1}$ (we are unaware any other humoral substance that increases to such an extraordinary degree). In general, in humans and in experimental animals, these levels correlate with the severity of the condition, and remain elevated for the duration of the inflammatory process (Steinwald *et al.*, 1999; Becker *et al.*, 2007). Patients with SIRS may have very high levels of serum ProCT, and they overlap with sepsis. However, the highest levels tend to occur in sepsis. Notably, the levels that occur in systemic viral infections usually are considerably lower than bacterial infection (Nylén *et al.*, 1996), but occasionally overlap (Thayvil *et al.*, 2005).

Fluids other than blood can also manifest increased levels of ProCT. For example, salivary levels of this prohormone are increased in periodontitis (Bassim *et al.*, 2008). Also, in persons with wartime extremity injuries, the ProCT in the wound exudate is significantly increased in those patients whose wounds dehisce when compared with wounds that subsequently heal (Forsberg *et al.*, 2008). Serum ProCT levels

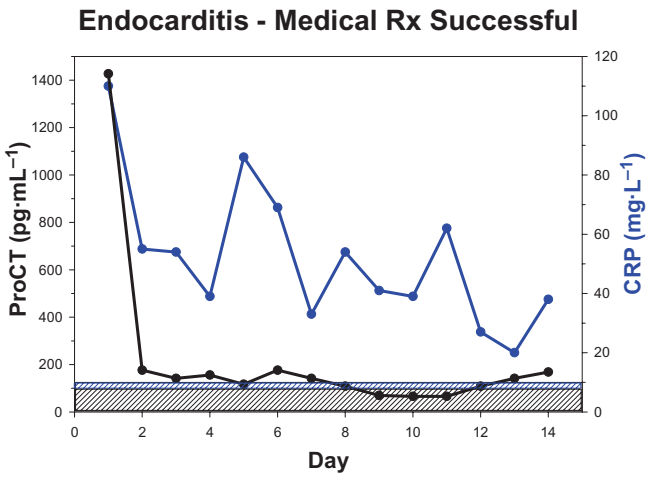

Figure 3 Serial mean serum procalcitonin (ProCT) levels from 19 patients with bacterial endocarditis (Duke classification) who were successfully treated with antibiotics. Within 1 day, ProCT levels had decreased markedly and these values remained close to normal. However, the CRP decline was much more gradual (unpublished data in collaboration with Dr Jonathan Sandoe, Leeds, Great Britain).

below 500 pg·mL^{-1} are relatively uncommon in patients with classic sepsis symptomatology, but values below this level may indeed occur (Becker *et al.*, 2007). Clinically, the daily determination of ProCT in sepsis is most useful (Becker *et al.*, 2007; Castelli *et al.*, 2009; Hochreiter *et al.*, 2009). During the course of a septic process, there may be a marked increase in serum ProCT, often indicating an exacerbation of the illness. Moreover, a decreasing level often is a favourable sign (Figure 3) (Jensen *et al.*, 2006). However, it should be emphasized that during the course of a septic process, complications may occur, such as hypotension, shock, heart failure, respiratory insufficiency or disseminated vascular coagulation. These conditions greatly influence the course and ultimate outcome of the disease without necessarily, in themselves, affecting ProCT levels. Moreover, the eventual outcome is influenced by the precipitating cause, as well as the clinical care. Thus, clinical severity-of-illness scores or prognostic scores, some of which involve parameters such as age or concomitant illness, for example, Acute Physiology and Chronic Health Evaluation score (Whang *et al.*, 1998; Claeys *et al.*, 2002), multiple organ failure (MOF) scores (Hensler *et al.*, 2003), sequential organ assessment score (Castelli *et al.*, 2004) or simplified acute physiology score II (Cheval *et al.*, 2000) often correlate with serum ProCT levels, albeit only approximately.

Initiators of hyperprocalcitonaemia

Studies of the initiation and the pattern of response of hyperprocalcitonaemia have been performed in experimental animals (Morgenthaler *et al.*, 2003) and in humans (Dandona *et al.*, 1994). In healthy human volunteers who were administered a single dose of endotoxin (lipopolysaccharide, LPS), serum levels of ProCT increased within 3 h, peaked within 24 h and then slowly declined (Preas *et al.*, 2001). Moreover, high levels persisted, and remained above baseline for at least 7 days. In some instances, they did not normalize until 2 weeks. In contrast, in various laboratory and clinical settings, the classic cytokine markers have been found to be very evanescent (Thijs and Huck, 1995; Becker *et al.*, 2004) and often exhibit marked inter-individual variations (Bone, 1996).

The primary pathophysiological trigger for the increase of serum ProCT is 'infection' (whether exogenous in origin or via endogenous translocation of bacterial toxins across the gut wall or other epithelial barriers). This often results in the appearance in the circulation of LPS, although it is likely that other constituents of microorganisms also are offenders. Soon thereafter, there is a secondary release of the putative principal pro-inflammatory and anti-inflammatory cytokine messengers. More study is required, and other messenger molecules may become identified, but current data indicate that mediators such as TNFα (as well as IL-1β and IL-6) comprise the specific proximate stimuli to hyperprocalcitonaemia (Redl *et al.*, 2000, 2001; Whang *et al.*, 2000; Domenech *et al.*, 2001; Preas *et al.*, 2001). Although there may be recurrent elevations of these cytokines during the course of sepsis, their short-lived duration and their erratic fluctuations differ markedly from the long-term persistence of hyperprocalcitonaemia, a 'late' and long-lasting marker and mediator.

When the secretion of ProCT becomes ubiquitous

As previously mentioned, secretion by the C cells of the thyroid, the pulmonary neuroendocrine cells of the lungs and perhaps other gastrointestinal neuroendocrine cells constitute the principal sources of serum ProCT in the healthy subject. Here, these cells produce CT from ProCT, the former being stored in dense-core secretion granules within the cytoplasm. These granules release their mature peptide contents when the appropriate stimulus appears at the cell surface, that is, a 'regulated' secretion (Burgess and Kelly, 1987).

It has been reported that serum immunoreactive CT moieties persist in the serum of humans in spite of their having had a prior thyroidectomy (Silva *et al.*, 1978). In humans, and also in thyroidectomized monkeys, small but measurable amounts of such immunoreactivity was extracted from many tissues (Becker *et al.*, 1979; 1980), and low levels of the mRNA of the CT gene, CALC 1, were later found (Russwurm *et al.*, 2001). However, in sepsis, reverse transcriptase polymerase chain reaction studies revealed considerable amounts of CALC I mRNA expression in nearly *all* tissues examined (fat, liver, lung, muscle, stomach, kidney, brain, etc.) (Müller *et al.*, 2001). Moreover, *in situ* hybridization studies demonstrated that multiple cell types within tissues participated in this up-regulation (Linscheid *et al.*, 2003). Because of the huge mass of fat in the body, this phenomenon was studied *ex vivo* and *in vitro* with human fat cells. It was reported that the addition of LPS to these cells induced a large increase of both CALC I mRNA and hormonal secretion. Analogous increases were also produced by TNFα and IL-1β (Linscheid *et al.*, 2003). This ubiquitous expression of ProCT in nearly all tissues appears to be a unique phenomenon. In essence, the body becomes an endocrine gland, secreting ProCT in an ongoing unregulated constitutive fashion (Burgess and Kelly, 1987). It has been postulated that this sepsis-related increase of CALC-I gene transcription is mediated via stimulus-specific response elements within the promotor of the gene. In this respect, the

term 'hormokine' has been proposed; that is, the cytokine-like behaviour of a hormone (i.e. ProCT) during inflammation or infection (Müller *et al.*, 2001; Nylén and Alarifi, 2001; Müller 2007). Because very little, if any, mature CT hormone is produced in sepsis, it can be hypothesized that non-neuroendocrine tissue lacks the enzymatic potential to adequately process and activate the immature peptide.

Immunoasssay of ProCT

In the medical literature, six assays for ProCT have been employed, three of which are available commercially. The authors have evaluated the performance of all of them, the characteristics of which are detailed in Table 1.

Currently, there is no available assay that detects ProCT exclusively. Initially, for the purpose of studying systemic infection, inflammation and sepsis, a very sensitive, single-antibody assay of NProCT was developed for research purposes (Nylén *et al.*, 1995; Snider *et al.*, 1997). This assay detects ProCT and the free NProCT peptide. All other assays detect ProCT and the free conjoined CT: CCP-I peptide, utilizing two antibodies in a sandwich assay.

When ProCT is high in patients with systemic infection or sepsis, with the exception of CT, all of the peptides shown in Figure 4 increase, albeit not to the same extent, and the pattern varies from patient to patient and at varying times during the illness. In spite of this phenomenon, on a practical level, the clinical utility of the assays is not compromised. For simplicity of expression, investigators utilizing these assays refer to the material being measured as 'procalcitonin'.

A very promising development in assays for ProCT is the recently available Kryptor procedure (Steinbach *et al.*, 2004; Becker *et al.*, 2007). Following preparation with calibration using control samples, the ProCT of a serum sample can be determined in 20 min, with excellent intra- and inter-assay precision. Although it should be recognized that the mean level for healthy controls is less than the functional sensitivity of the Kryptor assay (60 $pg \cdot mL^{-1}$), this instrument can still detect modest fluctuations from day to day, distinguish meaningful changes in ProCT (e.g. those changes associated with significant infection) and also perform multiple determinations at a cost that is comparatively low (see footnote to Table 1 for a more in-depth discussion of ProCT assays). It is now being utilized successfully to aid in the diagnosis of sepsis, and to follow the cause of this illness, as well as other severe infections such as pneumonia (Müller *et al.*, 2007). Its reliability and sensitivity lend itself to the detection of the mild elevations that occur in a bacterial infection very early in the appearance of fever in infants (Dauber *et al.*, 2008; Maniaci *et al.*, 2008); it also offers useful diagnostic information in children with acute respiratory infection (Schützle *et al.*, 2009). Other uses have been for identification of patients with paralytic ileus, the levels of whom are close to normal, as opposed to those with ileus of an obstructive or vascular causation (Maruna *et al.*, 2008). Furthermore, this instrument should be able to detect the sudden increases of ProCT that herald the onset of systemic infection in patients with an intravascular catheter (Figure 5).

Perhaps the most exciting benefit from ProCT measurements obtained by Kryptor technology is its use as a marker to reduce overutilization of antimicrobial therapy. Throughout the world, the needless or excess administration of antibiotics has led to a marked increase of drug-related morbidity, immunological sensitization and expense, and, above all, has resulted in the emergence of drug-resistant bacteria on a massive scale (Patterson, 2001; Cetinkaya and Cag, 2004; Cohen, 2007; Cameron and Maling, 2008). In this respect, a series of publications have shown the value of ProCT-guided parameters in decreasing unnecessary antibiotic therapy or its duration of administration (Christ-Crain *et al.*, 2004; 2006; Stolz *et al.*, 2007; Briel *et al.*, 2008; Nobre *et al.*, 2008; Schroeder *et al.*, 2009).

Because most acute respiratory infections are viral, the routine antibiotic therapy for this condition, that is, so commonly encountered in primary care settings, offers very little benefit (Briel *et al.*, 2008). Nevertheless, it is likely that viral infections compose the greatest use of antibiotics (i.e. employed in over half of such cases). Recently, in a large study, 53 primary care physicians recruited 458 adults with acute respiratory infections who were thought to be in need of antibiotics (Briel *et al.*, 2008). They were then randomly assigned to ProCT-guided therapy or standard therapy. The guidelines were: ProCT serum level < 100 $pg \cdot mL^{-1}$ – antibiotics strongly discouraged; ProCT 100–250 $pg \cdot mL^{-1}$ – antibiotics discouraged; ProCT > 250 $pg \cdot mL^{-1}$ – antibiotics recommended. Follow-up examinations were weekly for 1 month. There was no difference in number of days missed from work, and no difference in ongoing infection or relapse. The ProCT-guided group had a prescription rate that was significantly lower than the standard therapy group.

Utilizing a ProCT guidance strategy, similar impressive diminutions of antibiotic treatment have been demonstrated in hospitalized patients with community-acquired pneumonia (Christ-Crain *et al.*, 2006) and in exacerbations of chronic obstructive pulmonary disease (Stolz *et al.*, 2007), and may have shortened the duration of treatment in patients with sepsis (Nobre *et al.*, 2008; Hochreiter *et al.*, 2009). Thus, in addition to its role in assisting in the diagnosis and follow-up of respiratory diseases (Müller *et al.*, 2007), the measurement of ProCT shows much hope as a weapon against antibiotic overusage.

The toxicity of ProCT in sepsis

Toxicity of ProCT in vivo

To date, all of the physio-pharmacological studies that have been performed to evaluate the effects of ProCT have indicated potentially harmful effects. Based on multiple prior studies revealing that ProCT metabolism in hamsters was very similar to that of humans, a model for virulent septic peritonitis was developed, utilizing the intraperitoneal placement of agar pellets containing premeasured quantities of *Escherichia coli* (Nylén *et al.*, 1998; Steinwald *et al.*, 1999). The 72 h mortality of the hamsters, as well as serum levels of ProCT, correlated well with the number of bacteria administered. The bacterial dose was then adjusted to achieve a mortality of approximately 50%. Subsequently, ProCT that had been shown previously to be non-toxic in normal animals, was injected intraperitoneally into septic hamsters. In repeated

Table 1 Characteristics of procalcitonin (ProCT) assays (as determined in the authors' laboratory)

Assay	*Source*	*Type of test*	*Status*	*Peptides identified*	*Low assay standard* ($pg \cdot mL^{-1}$)	*Functional sensitivity* ($pg \cdot mL^{-1}$)	*Mean of healthy controls*[a] ($pg \cdot mL^{-1}$)	*Assay time*	*Comments*
NProCT	Becker *et al.*	ELISA	Research	ProCT and NProCT	10	20	33[b]	16–18 h	Highly sensitive, quantitates most normals; equipment readily available Slow; requires plate reader
ProCa-S	BRAHMS	ILMA	Research	ProCT and CT : CCP-I	5	20	31	3 h	The most sensitive ILMA assay, rapid assay Requires luminometer
PCT sensitive	BRAHMS	ILMA	Research	ProCT and CT : CCP-I	5	50	13	3 h	Highly sensitive; rapid Requires luminometer
QPCT	BRAHMS	Solid-phase	Commercial	ProCT and CT : CCP-I	(500)	(500)	(500)	5 min	Rapid bedside test needing no equipment Semiquantitative test; provides determinations only within very broad ranges
LUMItest	BRAHMS	ILMA	Commercial	ProCT and CT : CCP-I	80	500	235	2 h 45 min	The most widely used test to date; rapid Relatively insensitive; cannot detect mild elevations or mild variations; expensive to do single determinations; requires luminometer
KRYPTOR	BRAHMS	TRACE	Commercial	ProCT and CT : CCP-I	20	60	53	Initial sample: 50 min Stat sample: 25–45 min[c]	Sufficiently sensitive for most purposes; readily detects daily changes, ideal for a large number of determinations; and hence inexpensive for this usage Most appropriate for large-volume usage as in hospitals, large infectious disease services, intensive care units or emergency rooms Requires Kryptor machine

Note: As shown above, no currently available commercial assay measures exclusively procalcitonin. All assays detect at least one other constituent of this prohormone. High-performance liquid chromatography studies of calcitonin gene peptides extracted from concentrated normal human serum reveal very low levels of ProCT (2.5 $pg \cdot mL^{-1}$) plus its component peptides (NProCT, CT, CCP-I and CT : CCP-I) (Snider *et al.*, 1997). In hyperprocalcitonemic states, procalcitonin and its constituent peptides all may be increased to varying extent from patient to patient. Other than the hyperprocalcitonaemia that is due to secretion from a neuroendocrine tumour, the amidated free CT in patients with sepsis, infection or systemic inflammation remains low. CT is not measurable in any of the above assays. For simplicity of expression, all these above assays are now referred to by investigators as 'procalcitonin' assays.

[a]The discrepancy between functional sensitivity and healthy control values (i.e. LUMItest, PCT sensitive, KRYPTOR) implies considerable uncertainty of the values in this population. Because none of these cited assays are capable of measuring the actual values of ProCT in sera from healthy controls, the authors recommend that investigators not try to make comparisons between data points below the functional sensitivity of the assays because such comparisons are likely to be unreliable.

[b]This level is 90% aminoprocalcitonin, based on chromatography studies. The actual mean level of ProCT in healthy controls is <4 $pg \cdot mL^{-1}$. ProCT + CT : CCP-I < 5 $pg \cdot mL^{-1}$ (Snider *et al.*, 1997).

[c]The initial sample time includes the time required for powering up the equipment; performing the daily maintenance; and running the calibrators, control samples and the first sample. During the week, the machine may be left on and the calibrators need to be run only once. The time required for daily maintenance is less than 20 min. Additional samples loaded into the Kryptor machine along with the first sample require 1–2 min per sample; thus, for example, five additional samples can be measured within 5–10 min. The stat sample time shown above is the time required to measure a single sample that is loaded by itself after starting the machine. This includes the time that may be required for the automatic dilution and recount of a sample which contains a concentration of ProCT > 50 $ng \cdot mL^{-1}$.

ProCT, procalcitonin; NProCT, aminoprocalcitonin; CT : CCP-I, the conjoined peptide consisting of calcitonin + calcitonincarboxypeptide I (see Snider *et al.*, 1997); ELISA, enzyme-linked immunosorbent assay; ILMA, immunoluminometric assay; TRACE, time-resolved amplified cryptate emission.

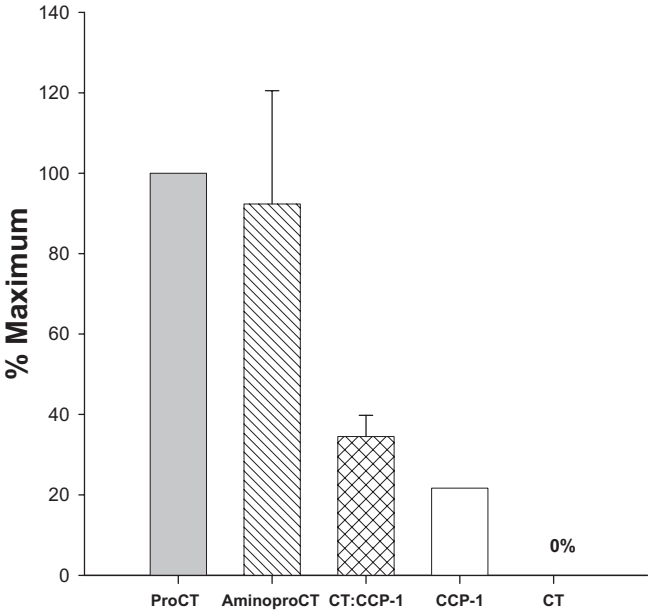

Figure 4 Mean peak concentrations of constituent peptides of procalcitonin (ProCT) of several patients with sepsis (G-75 Sephadex gel filtration and C_{18} reversed-phase high-performance liquid chromatography). The data are expressed in terms of the percentage of the mean peak level of the intact ProCT. In sepsis, some of the ProCT appears to have a two-amino acid truncation at the amino terminus (Weglöhner *et al.*, 2001). The free, amidated, mature calcitonin remains absent or low, because the constitutive secretion of ProCT apparently lacks the enzymatic processing to cleave this peptide from the prohormone.

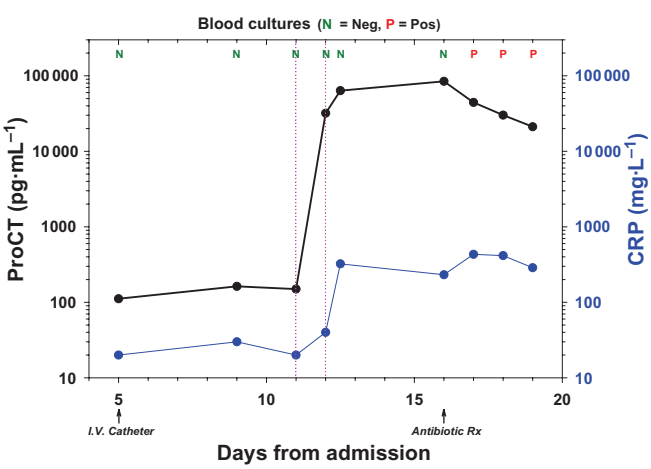

Figure 5 Study of a trauma patient admitted to the intensive care unit who had an intravascular catheter inserted at day 5 following hospitalization with a consequent risk of ensuing infection (see Maki *et al.*, 2006). Antibiotics were started at day 16 because of the onset of fever, and the catheter was removed a day later. Retrospective studies revealed that on the morning of day 11, procalcitonin (ProCT) suddenly increased from a value that was close to normal (120 pg·mL^{-1}) to 25 000 pg·mL^{-1}, and by that evening had increased further to 60 000 pg·mL^{-1}. However, on the same morning, the CRP had only slightly increased, and then subsequently increased further. Blood cultures remained negative (N) until day 17, when they became positive (P). Thus, the initial alarming increase of serum ProCT had occurred 5 days prior to the clinical diagnosis of catheter-related sepsis.

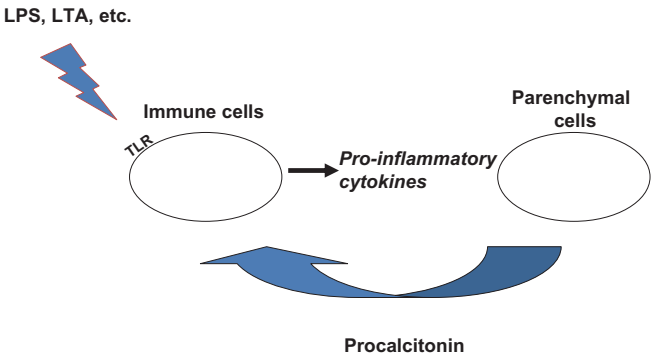

Figure 6 Illustrative sequence of events by which endotoxin (lipopolysaccharide) from Gram-negative bacteria or other bacterial products, such as lipotechoic acid (LTA) from Gram-positive bacteria (Ryu *et al.*, 2009), interact with immune cells via toll-like receptors (TLRs) to initiate a pro-inflammatory cytokine response that induces the systemic hypersecretion of procalcitonin (ProCT) from parenchymal cells. In turn, in a feedback manner, the blood cell production of these same cytokines may further augment the local levels of ProCT.

experiments, this resulted in mortalities close to 100% (Nylén *et al.*, 1998).

ProCT induces proinflammatory-like effects on leukocytes

In a manner similar to the inflammatory action of LPS, ProCT increased the expression of surface markers on human neutrophils and lymphocytes (CD16 and CD14, respectively) (Wei *et al.*, 2008). This is thought to reflect the motion of intracellular secretory vesicles towards the cell surface. In this study, ProCT also increased the concentration of intracellular calcium ions, similar to the action of the proinflammatory cytokine, IL-8. In another study, ProCT decreased both phagocytic and candidacidal activity of neutrophils in a dose-dependent manner (Pincikova *et al.*, 2005). In agar plate studies, ProCT also inhibited the microbicidal effect of serum and blood upon cultured *E. coli*. This prohormone also suppressed the blastic transformation of activated T lymphocytes of normal human blood, but increased the activity of unstimulated autologous lymphocytes (Bucova *et al.*, 2006).

ProCT increases leukocyte-derived cytokines

In recent experiments, human recombinant human ProCT was added to whole human blood, as well as to human peripheral blood lymphocytes that had been treated by gradient centrifugation and incubated (Liappis *et al.*, 2007a). In whole blood, there was a marked dose-dependent increase of pro-inflammatory cytokines above control levels. Among the isolated lymphocytes, TNFα was found to be the highest responder. Thus, TNFα, a potent stimulant of ProCT production (Whang *et al.*, 2000; Redl *et al.*, 2001) may, along with other cytokines, further reinforce the already high ProCT levels of sepsis in a self-perpetuating cascade fashion (Figure 6).

ProCT effects on leukocyte migration

Polymorphonucleocytes mediate much of the complex cytokine and inflammatory responses that occur in infection and

sepsis (Wagner and Roth, 1999; Martins *et al.*, 2006). In a study, these cells were separated from the heparinized blood of normal human volunteers and were incubated at 37°C with recombinant human ProCT at several dilutions (Liappis *et al.*, 2007b). The chemotactic index was calculated by comparing migration towards the chemoattractant, formyl methionyl leucylphenylalanine peptide. A dose-dependent decrease in the chemotactic index was repeatedly found. These findings suggest that the marked malfunction of neutrophils that is known to occur during sepsis (Egger *et al.*, 2004; Alves-Filho *et al.*, 2008) is in part due to ProCT. Interestingly, a similar inhibition of migration towards a chemoattractant has been noted for mononuclear lymphocytes (Wiedermann *et al.*, 2002). This diminution of chemoattractant stimulation may conceivably impact upon macrophage activation, as well as on phagocytosis. The effect on these cells appears to involve an interaction of ProCT with a cyclic AMP-dependent protein kinase A.

ProCT augments nitric oxide

Increased levels of the vasodilator, nitric oxide (NO), occur in sepsis, and these levels correlate with severity (Brealey *et al.*, 2002; Mitaka *et al.*, 2003). Particularly high values are found in patients with complicating hypotension (i.e. severe sepsis, septic shock). Studies with cultured rat aortic vascular smoth muscle cells have documented that ProCT alone does not raise inducible NO synthase (iNOS) gene expression. However, if these are previously primed with LPS plus TNFα and interferon-γ in order to institute a pro-inflammatory stimulus, the later addition of ProCT strikingly augments iNOS and NO levels (Hoffmann *et al.*, 2002). Thus, as is the case for *in vivo* studies, ProCT itself is not primarily an initial pro-inflammatory stimulus. Instead, it is a potent amplifier of the inflammatory cascade.

Impact on energy homeostasis

As discussed, ProCT contains an aminoterminal peptide, NProCT. Following processing, this segment also is found free in the blood, having been cleaved from the prohormone by a convertase enzyme. It was reported that a single intracerebroventricular administration of NProCT in rats induces a significant decrease of food intake and weight over a period of over 48 h. It also increases body temperature and locomotor activity, and appears to disrupt the integration of hormonal signals that may affect hypothalamic–pituitary energy homeostasis (Tavares *et al.*, 2007). Curiously, ProCT has been alleged to be located within the normal hypothalamus by immunostaining, although it is not otherwise known to be stored in cells (Ojeda *et al.*, 2006). Moreover, the administration of LPS in the rat was noted to induce CT gene expression in the neighbouring pituitary (Kiriyama *et al.*, 2002). Whether these phenomena play a role in the clinical manifestations of sepsis remains to be determined.

ProCT is a blocker of CT-gene-related peptide (CGRP) activity

ProCT derives from one of a family of genes. The gene in question possesses an alternative splice variant, CT-gene-related mRNA, which gives rise to CGRP, a hormone possessing a slight homology with CT (Amara *et al.*, 1982; Becker *et al.*, 2002). CGRP normally functions as a peptidergic agent and is involved in various aspects of neurotransmission. In the healthy, non-infected state, there is a preferential synthesis of either CT mRNA or CGRP mRNA, according to ambient conditions and perhaps neuroendocrine cell phenotype. Similar to the case for CT mRNA in sepsis or other varieties of severe infection and systemic inflammation, there is also a tissue-wide constitutive expression of CGRP mRNA (Domenech *et al.*, 2001). As a result, CGRP is increased in sepsis (although not to the same extent as is ProCT) (Linscheid *et al.*, 2005).

CGRP exerts effects that are anti-inflammatory and that would be of potential benefit in sepsis (i.e. increased phagocytosis, down-regulation of TNFα, dilatation of coronary arteries, positive cardiac inotropic and chronotropic effects, antibacterial actions, etc.) (Dong *et al.*, 1993; Ichinose and Sawada, 1996; Sheykhzade and Berg Nyborg, 2001; Okajima and Harada, 2006; El Karim *et al.*, 2008; Li *et al.*, 2008). Both CT and CGRP act via a group of receptors that are formed by complexes between the receptors ('calcitonin receptor' and 'calcitonin receptor-like receptor') and one of three receptor activity-modifying proteins (RAMPs) (McLatchie *et al.*, 1998; Poyner *et al.*, 2002). It was hypothesized that in sepsis, ProCT might blunt the actions of CGRP at its receptor site. Accordingly, the biological activity of ProCT upon this group of human receptors was explored. These receptors were transiently expressed into COS-7 cells, alone or together with individual RAMPs, so as to generate receptors for CGRP (Sexton *et al.*, 2008). Subsequently, ProCT was examined for its ability to influence the action of exogenous CGRP on its receptor, as assessed by intracellular cyclic adenosine monophosphate (cAMP) accumulation. In repeated experiments, using concentrations comparable to those observed during sepsis, there was a marked inhibition of the CGRP response at its specific receptor. Interestingly, a study in which septic animals were treated with CGRP reported a marked increase of survival when CGRP was administered before endotoxemic shock was produced (Gomes *et al.*, 2005). Importantly, this same therapy was unsuccessful if administered with a CGRP receptor antagonist. These studies strongly suggest that the hyperprocalcitonaemia occuring in sepsis blocks the effects of CGRP and prevents its action. The demonstration of the strong attenuation of the action of CGRP at its receptor provides an insight into one of the several mechanisms underlying the ProCT toxicity in sepsis and its related conditions (Christ-Crain and Müller, 2008; Sexton *et al.*, 2008).

Immunoneutralization of ProCT in sepsis

Hamsters

Based on clinical and *in vivo* animal studies, as well as many *in vitro* findings, it is apparent that ProCT *per se* is a toxic factor in sepsis that adversely influences survival. Accordingly, a goat antiserum was raised to the midportion of ProCT, that is, a segment of the immature non-amidated CT that is cross-reactive with the prohormone (Figure 2). The administration of this antiserum markedly increased survival of

hamsters. Importantly, this beneficial effect occurred both when the immunoneutralization was performed at the initiation of sepsis, as well as after its onset (Nylén *et al.*, 1998).

Pigs

To further investigate in detail the physiological and metabolic consequences of sepsis, to evaluate how ProCT might affect these parameters and to determine whether survival would be increased, a large animal model of a rapidly fatal, porcine polymicrobial sepsis was developed during a series of experiments (Martinez *et al.*, 2001; Wagner *et al.*, 2002; Becker *et al.*, 2003). It was also desirable to evaluate whether such immunoneutralization of the harmful effects of sepsis could be achieved by an antiserum raised to another portion of the ProCT molecule. For this purpose, an antiserum was produced in rabbits that was specific to the aminoterminus portion of this prohormone. A highly virulent sepsis was induced in adult male Yorkshire pigs by the intraperitoneal instillation of a suspension of cecal content (1 $g \cdot kg^{-1}$ bodyweight) that was accompanied by the further addition of a measured amount of *E. coli*. Within 4 h, all control animals were premorbid and manifested a symptomatology similar to the syndrome of MOF that occurs in humans with preterminal sepsis, that is, acidosis, renal failure, cardiac failure and shock. (Becker *et al.*, 2003).

In these experiments, highly purified ProCT-reactive rabbit IgG was administered intravenously, and the control animals received non-ProCT-reactive IgG. All animals had physiological data determined (e.g. urine output, core body temperature, arterial pressure, heart rate, cardiac index, stroke index), as well as metabolic data (e.g. blood urea nitrogen, serum creatinine, arterial lactate and pH). These were obtained and recorded hourly until death, or until the predetermined time of sacrifice, that is, 15 h after induction of sepsis. (At this latter time, all non-treated animals were dead.)

In these studies, which were repeated several times, the immunoneutralization of ProCT resulted in amelioration or stabilization of several of the physiological and metabolic data. Importantly, both early immunoneutralization (i.e. concomitant with induction of sepsis) or late immunoneutralization (i.e. when the animals were deemed to be premorbid), greatly reduced short-term mortality. In early immunoneutralization, none of the control animals survived, while the survival of the treated animals was 85% (Wagner *et al.*, 2002). For late immunoneutralization, there were no survivors for the controls, while 80% of the treated pigs survived (Martinez *et al.*, 2001).

Thus, in studies involving two species of animals, immunoneutralization of ProCT improved both the symptomatology and the survival of highly virulent sepsis. In pigs, this occurred not only early in the course of the illness, but also after the advent of a very advanced septic state. Hence, immunoneutralization of ProCT may be useful in treating well-established sepsis and, perhaps, also in preventing the possible occurrence of this complication in non-septic patients with severe infections.

Comments and conclusions

Several characteristics of a marker–mediator of sepsis that would be most applicable to a successful therapy by immunoneutralization in the human are shown in Table 2. Few sepsis markers–mediators have met all or even half of these features (e.g. endotoxin, TNFα, IL-1, enterobacterial common antigen) (Seam and Suffredini, 2007). However, ProCT meets all of them. Therefore, therapeutic immunoneutralization of this prohormone merits serious consideration.

Table 2 Characteristics of a marker–mediator of sepsis that would be most applicable to a succesful therapy by immunoneutralization

1. A toxic marker that is elevated in most patients with sepsis
2. A correlation between the titre of the serum marker and the severity and outcome of sepsis
3. Demonstration that the marker is a toxic mediator
4. Persistence of the marker for a sufficiently long duration to allow for a suitable window for appropriate therapy
5. A rationale for clinical trials that is based upon results in animals having a bacterial-induced sepsis that is analogous to that occurring in humans
6. Study of more than species of experimental animal
7. A marker that is measurable with sensitivity, reproducibility and specificity. Accordingly, a presumptive diagnosis of sepsis that was based only on clinical criteria could be confirmed prior to a therapeutic trial.
8. As a corollary, the selection and/or triage of the candidate patients would be based upon a rapid determination of the blood level of the marker. Moreover, such a marker-based diagnosis and a marker-driven selection might greatly reduce the number of patients needed to be enrolled for a clinical trial.

Multiple studies have demonstrated that serum levels of ProCT are markedly increased in humans with sepsis, severe infection and severe inflammation. The high levels last as long as the inflammatory process persists, and tend to correlate with the outcome of the illness. ProCT was found to be toxic to septic animals, and in vitro studies of this prohormone also documented several noxious effects. Therapeutic immunoneutralization of animals with severe sepsis has been proven successful in two species. Such findings strongly indicate that ProCT immunoneutralization in humans with these conditions offers considerable promise. Moreover, the rapid onset of increased serum ProCT with the advent of the illness and the very long-lasting duration of this elevation provide a broad clinical window for therapeutic intervention. Furthermore, the ease and rapidity of ProCT measurement allow for a swift documentation of the presence of the illness, and permit the selection and stratification of the cases to be treated. Conceivably, not only sepsis, but also SIRS might be amenable to such therapy. These multiple studies provide a groundwork for clinical pharmacotherapy trials with engineered humanized monoclonal antibodies.

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