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Research paper

Isolation and characterization of pharmaceutical grade human pentraxins, serum amyloid P component and C-reactive protein, for clinical use

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ABSTRACT

The human pentraxin proteins, serum amyloid P component (SAP) and C-reactive protein (CRP) are important in routine clinical diagnosis, SAP for systemic amyloidosis and CRP for monitoring the non-specific acute phase response. They are also targets for novel therapies currently in development but their roles in health and disease are controversial. Thus, both for clinical use and to rigorously elucidate their functions, structurally and functionally intact, pharmaceutical grade preparations of the natural, authentic proteins are required. We report here the production from normal human donor plasma and the characterization of the first such preparations. Importantly, we demonstrate that, contrary to reports using recombinant proteins and less well characterized preparations, neither CRP nor SAP stimulate the release by human peripheral blood mononuclear cells *in vitro* of any TNFα, IL-6 or IL-8, nor does SAP cause release of IL-1β or IL-10. Furthermore neither of our preparations was pro-inflammatory in mice *in vivo*.

1. Introduction

The human pentraxin proteins, serum amyloid P component (SAP) (Pepys et al., 1997) and C-reactive protein (CRP) (Pepys and Hirschfield, 2003), are normal circulating plasma proteins which are important in routine clinical diagnosis. They are also targets for novel therapies currently being developed for major diseases (Pepys et al., 2002, 2006; Kolstoe et al., 2009; Bodin et al., 2010; Gillmore et al., 2010). However some of their putative roles in health and disease are controversial. Comprehensively validated, highly purified, authentic, native, structurally intact and fully functional human SAP, isolated from normal human plasma, is essential for SAP scintigraphy (Hawkins et al., 1988b,

Abbreviations: BVDV, bovine viral diarrhea virus; cGMP, current good manufacturing practice; CRP, C-reactive protein; ELISA, enzyme linked immunosorbent assay; ESIMS, electrospray ionization mass spectrometry; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IBRV, infectious bovine rhinotracheitis virus; IS, international standards; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; SAA, serum amyloid A protein; SAP, serum amyloid P component.

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1990a, 1990b) in patients with amyloidosis (Pepys, 2006). Human CRP of comparable quality and authenticity is also necessary, for both critical experimental studies *in vitro* and *in vivo* studies in normal human volunteers, to rigorously establish its functional effects. Material for human use *in vivo* must be of pharmaceutical quality, produced under conditions compliant with current standards of current good manufacturing practice (cGMP), in order to be acceptable to ethical and regulatory authorities. We report here the production and characterization of the first such preparations.

Human SAP is universally present in all amyloid deposits (Pepys et al., 1997) as a result of its avid calcium dependent binding to all types of amyloid fibrils (Pepys et al., 1979b), regardless of their protein composition. We utilized this property in our invention of radiolabeled SAP scintigraphy for the safe, non-invasive diagnosis and monitoring of amyloid deposits in systemic amyloidosis (Caspi et al., 1987; Hawkins et al., 1988b, 1990a, 1990b). This method revealed much of the previously obscure natural history of the different forms of systemic amyloidosis, including the critical fact that amyloid deposits can regress when the abundance of the respective amyloid fibril precursor protein is substantially reduced (Hawkins et al., 1993a, 1993b; Holmgren et al., 1993; Hawkins, 1994). These observations have underpinned major advances in diagnosis and management of amyloidosis and led to much improved patient survival, especially in the UK National Health Service National Amyloidosis Centre which is directly funded by the UK Department of Health to provide diagnostic and management advisory services for the whole UK national caseload. The Centre follows the largest and most diverse cohort of systemic amyloidosis patients in the world, currently sees more than 3000 patients and performs about 1000 SAP scintigraphy examinations annually. The investigation requires intact, native, highly purified, clinical GMP grade human SAP for labeling with ^{123}I and intravenous injection into the patient. Unrelated to its use in diagnosis and monitoring, SAP contributes to the pathogenesis and/or persistence of amyloid deposition *in vivo* and is the target of novel therapeutic approaches to elimination of amyloid deposits which we have invented and are developing for clinical testing in collaboration with GlaxoSmithKline (www.pentraxin.com) (Pepys et al., 2002; Kolstoe et al., 2009; Bodin et al., 2010; Gillmore et al., 2010).

The physiological functions of neither human SAP nor CRP are fully understood but the most robust and reproducible observations indicate that they contribute to innate immunity against some bacterial infections. We have demonstrated this for smooth Gram negative bacteria with SAP (Noursadeghi et al., 2000) and for pneumococci with CRP (Loeffler et al., 2009). The avid binding of SAP to DNA (Pepys and Butler, 1987) and chromatin (Butler et al., 1990) strongly suggests that SAP may play a role in the appropriate, safe handling of these materials *in vivo*. More controversially it has been reported that SAP has an anti-fibrotic effect, for which several different mechanisms have been claimed, most recently via stimulation of IL-10 production (Castaño et al., 2009). There is even more wide ranging controversy over possible biological roles of human CRP, which has been claimed to be pro-inflammatory, cytokine stimulating, pro-atherogenic and pro-thrombotic (Ballou and Lozanski, 1992; de Maat and Trion, 2004; Labarrere and Zaloga, 2004; Bisoendial et al., 2005, 2007a, 2007b, 2009). However human SAP is a constitutive plasma protein with a circulating concentration in the range of about 20-50 mg/L (Nelson et al., 1991) which is tightly regulated and almost constant in each individual. In contrast, human CRP is the classical, highly dynamic, rapidly responsive, entirely non-specific acute phase protein with a 10,000 fold concentration range of about 0.05 to over 500 mg/L (Shine et al., 1981; Pepys and Hirschfield, 2003). Neither of these behaviors is consistent with a role in regulation of cytokine production and there is absolutely no clinical evidence in humans or experimental evidence in animals that endogenously produced high human CRP concentrations are inherently pro-inflammatory. There are also compelling, well controlled, rigorous *in vitro* and *in vivo* studies which show no stimulation of cytokine production by the pentraxins (Hirschfield et al., 2003, 2005; Gillmore et al., 2004; Pepys, 2005; Pepys et al., 2005; Taylor et al., 2005; Taylor and van den Berg, 2007; Tennent et al., 2008).

Most reports on pro-inflammatory effects of human CRP preparations have used inadequately characterized material isolated from human biological fluids or, more recently, commercial recombinant CRP produced in *E. coli*. The latter, manufactured only by the Oriental Yeast Company of Japan (Tanaka et al., 2002), is intended for use as an immunochemistry standard, and is sold by many different biochemical reagent companies. It is heavily contaminated with endotoxin and likely other bacterial products (Pepys et al., 2005). Although it has been claimed that a single gel filtration step removed all such contamination from this recombinant product (Bisoendial et al., 2005), experiments in two independent laboratories, using authentic, highly purified, very low endotoxin content, human CRP did not produce any pro-inflammatory effects *in vitro* or *in vivo* in mice (Pepys et al., 2005; Taylor et al., 2005). The reports claiming anti-fibrotic activity of SAP are also poorly controlled and/or otherwise flawed (Pilling et al., 2003; Haudek et al., 2006; Pepys et al., 2007; Tennent et al., 2007). Their conclusions are not supported by the complete absence of any abnormalities of connective tissue or fibrosis in patients on long term treatment with the SAP-depleting drug, CPHPC, in whom SAP values are persistently reduced by 90-99% (Gillmore et al., 2010), or in mice with either deletion of the SAP gene or transgenic expression of human SAP (Botto et al., 1997; Bickerstaff et al., 1999; Gillmore et al., 2004). In order to provide suitable reagents with which to resolve these various controversies we have isolated from the plasma of healthy individuals, pharmaceutical GMP grade preparations of human CRP and SAP and fully characterized them as contaminant-free and structurally and functionally intact.

2. Materials and methods

2.1. Plasma collection and testing by the Bio Products Laboratory Ltd

Plasma, derived exclusively from paid donors in the USA, was collected at centers approved by the UK Department of Health. Donor selection, donor examination and plasma collection were performed according to standards and/or requirements set by the UK Department of Health, in accordance with the European Pharmacopoeia monograph 'Human Plasma for Fractionation'. Every donation was tested and found non-reactive for: i)