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Traces of pFc' in IVIG interact with human IgG Fc domains and counteract aggregation

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ABSTRACT

To prevent multimer formation, intravenous immunoglobulin (IVIG) is often treated with traces of pepsin. So far, the mechanism behind this treatment has been unclear. Recently, we reported that human IgG4 binds other IgG molecules via Fc–Fc interactions. Here we show that IVIG treated with traces of pepsin (Nanogam) inhibits these interactions. We found that – besides IgG4 – peptides corresponding to IgG1 and IgG2 pFc' (products of limited pepsin digestion) are responsible for the inhibitory action. Using radiolabeled pFc', it was found that pFc' binds directly to IgG1. Furthermore, recombinant CH3 fragments were found to also possess binding activity, and potencies of inhibition varied over 3 orders of magnitude amongst the subclasses, IgG4 being most potent.

We propose that pFc' formation explains how limited pepsin digestion diminishes adverse effects of IVIG. In particular, the presence of this fragment can enhance the stability of IgG products including IVIG and therapeutical monoclonal antibodies. Indeed, using a model system it was found that acid-induced aggregation of IgG is reduced in the presence of pFc', suggesting a 'chaperone-like' activity of this fragment. Thus, pFc' can modulate Fc interactions and may therefore reduce adverse effects of IVIG, in particular by preventing oligomerization.

1. Introduction

Intravenous immunoglobulin (IVIG) consists of pooled IgG of more than 1000 donors. It was originally developed as antibody replacement therapy for immunodeficiencies, but is nowadays also widely used as immunomodulating agent in autoimmune and inflammatory diseases [1]. IVIG contains a wide range of antibody specificities, including alloreactive antibodies resulting in the formation of idiotypic–anti-idiotypic dimers [2]. Besides Fab-mediated IgG–IgG interactions, human IgG can bind to IgG via Fab–Fc interactions: antibodies directed against the Fc parts of IgG (rheumatoid factors) are found in sera of many rheumatoid arthritis patients. Furthermore, several studies describe the recognition of hIgG Fc by hIgG4, not via the usual antigen-binding regions, but via the Fc part of IgG4 [3–5]. We recently showed that whereas binding of IgG4 to IgG4 can be observed under near-native conditions, binding of IgG4 to IgG1 requires a conformational change of the latter [3]. This Fc binding activity of IgG4 could be inhibited by – amongst others – (unlabeled) IgG4 and polyclonal human IgG (intravenous immunoglobulin, IVIG). We found that one IVIG product (Nanogam) was able to inhibit this binding much better than expected on the basis of the IgG4 content. This led us to expect that these preparations contain material other than IgG4 that influences Fc binding.

Several IVIG products including Nanogam are treated at pH 4.4 with traces of pepsin [6]. This results in a product that contains no detectable amounts of multimeric or aggregated IgG and is well suited for intravenous administration. Limited pepsin digestion may contribute to viral safety [7] and is thought to selectively digest aggregates, or aggregate-prone, unfolded IgG [8,9], thereby preventing aggregation and diminishing adverse effects such as complement activation. However, the mechanism of action has not been clarified so far. Interestingly, conditions of the pepsin digestion (pH 4, 37 °C) appear to remove most aggregates also in the absence of pepsin [8,10]. Nevertheless, small traces of pepsin are added to prevent reaggregation [9]. Several studies showed that IVIG treated with traces of pepsin may have altered capacity to induce or mediate effects via its Fc part (effector functions). In particular, pepsin-treated IVIG inhibits antibody-mediated platelet

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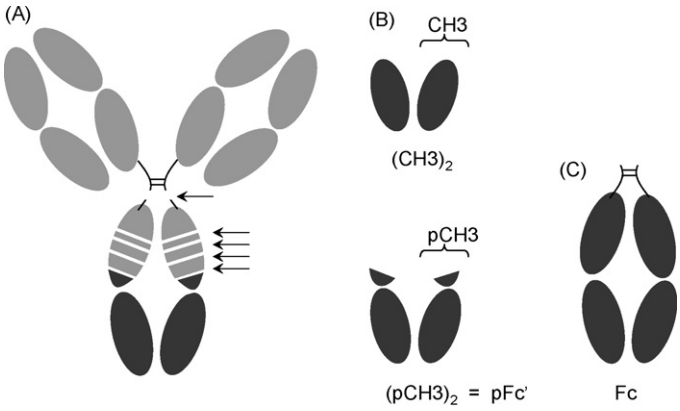

Fig. 1. (A) Pepsin digests IgG into F(ab)2, pFc' (black) and a number of smaller fragments. (B) Two CH3 or pCH3 domains form a non-covalent dimer in solution. Amino acid sequences of CH3 and pCH3 are indicated in Fig. 3F. (C) Papain digestion results in formation of an Fc fragment.

activation in the context of heparin-associated thrombocytopenia [11].

Because of the pepsin treatment, small amounts of F(ab)2 and pFc' fragment are present in Nanogam [12]. The pFc' fragment is almost equal to a CH3 dimer, as illustrated in Fig. 1. In this paper we show that pFc' fragments interact with IgG Fc. The pFc' fragments from IgG1, IgG2, and IgG4 all possess binding activity to IgG to different extents. This led us to formulate an alternative hypothesis in which the formation of pFc' rather than the digestion of aggregates is the mechanism by which pepsin contributes to reduction in aggregate formation in IVIG. We demonstrate that adding pFc' to IgG during acid-stress results in less aggregation, suggesting that pFc' possesses 'chaperone-like' activity for IgG. CH3–CH3 interactions are crucial for correct assembly and stability of IgG. The present results indicate that similar interactions play a role in aggregation and can be counteracted by introducing additional, isolated CH3 domains.

We will use the following nomenclature to indicate the various fragments (Fig. 1): CH3 = a single CH3 domain; $(CH3)_2$ is a dimer of CH3 as it is formed in solution; pCH3 is a single CH3 domain + 7 amino acids from the CH2 domain (derived from the pepsin-generated fragment); $(pCH3)_2$ = pFc' = a dimer of pCH3. The $(CH3)_2$ fragments used in this study are recombinantly produced. Fc fragments are the product of papain digestion. Furthermore, the term 'multimer' is used to indicate soluble aggregates of IgG.

2. Materials and methods

2.1. Inhibition assay

In this assay, IgG1 (adalimumab, Humira, Abbott) was coupled to CNBr-activated Sepharose (Amersham Biosciences, Uppsala, Sweden) at a density of 2.5 mg/100 mg Sepharose. 250 μL of a 2 mg/mL Sepharose suspension was incubated overnight with ^{125}I-labeled IgG4 Fc together with inhibitor in a total volume of 750 μL of PBS-AT (PBS, pH 7.4, supplemented with 0.3% bovine serum albumin, 0.1% Tween-20, 10 mM EDTA and 0.05% (w/v) NaN_3). After washing 5 times, binding was measured. Without inhibitor, binding was around 20% (expressed as the amount of radioactivity bound relative to the amount of radioactivity added). IgG4 Fc fragments were prepared by papain digestion as described previously [3]. IgG4 Fc was labeled by the ^{125}I chloramine-T method as described previously [13].

2.2. Fractionation of IVIG

2.2.1. Protein G affinity chromatography

IVIG (Nanogam, Sanquin, The Netherlands) contains 50 mg/mL of protein (>95% IgG), is formulated at pH 4, and contains 5% glucose. IMIG (Gammaquin, Sanquin, The Netherlands) contains 160 mg/mL of protein (>90% IgG), is formulated at pH 6.8, and contains 0.3 M glycine. IVIG or IMIG was layered to a column of 1 mL protein G Sepharose followed by PBS to obtain non-bound material. Bound material was eluted with 0.1 M glycine buffer pH 2.5. Fractions were neutralized immediately after collecting with 2 M Tris.

2.2.2. Size-exclusion chromatography

Samples (100 μl) were applied to a Superdex 200 HR 10/30 column (Amersham Biosciences, Uppsala, Sweden), which was connected to a HPLC system (ÄKTAexplorer) from Amersham Biosciences, Uppsala, Sweden. The column was equilibrated in PBS. For the estimation of protein size, the column was calibrated with the HMW calibration kit from GE Healthcare.

2.2.3. Anti-CH3 affinity chromatography

The non-bound fraction after protein G purification, as well as pooled low molecular weight fractions after SEC (Fig. 3), was further purified on a column of 1 mL Capture Select Human Fc affinity matrix (Nalgene). Nanobodies on this matrix bind to an epitope located in the CH3 domain of IgG. Elution of the bound fraction was performed with 0.1 M Glycine pH 3 or 2.5. Fractions were neutralized immediately after collecting with 2 M Tris, dialyzed against PBS and stored at 4 °C.

2.3. Limited pepsin digestion of IgG1, IgG2, and IgG4

1 mL of adalimumab (IgG1), omalizumab (IgG1, Xolair, Genentech/Novartis), panitumumab (IgG2, Vectibix, Amgen) or natalizumab (IgG4, Tysabri, Biogen Idec, Inc.) was digested at 4 mg/mL in 0.1 M acetate buffer pH 4.2 with pepsin (Sigma) 1:100 (w/w) at 37 °C. Time was varied between 0 and 1000 min; optimal yields of pFc' were obtained after 90 min (IgG1), 180 min (IgG2) or 20 min (IgG4) (based on SDS-PAGE or HP-SEC). Allotypes of adalimumab and omalizumab were Gm1(za) and Gm1(f), respectively (L.A. Aarden, personal communication). pFc' fragments were purified by removal of non-digested material using protein G affinity chromatography (pFc' in flow through), and subsequent removal of pepsin and $F(ab)_2$ using a-CH3 affinity chromatography (Capture Select Human Fc affinity matrix, Nalgene) as described above. For pFc' fragments from omalizumab and panitumumab this procedure yielded very low yields. Alternatively, pFc' fragments were purified by HP-SEC.

2.4. Recombinant CH3 fragments of IgG1 and IgG4

The CH3 regions of IgG1 and IgG4 (pEE, Lonza Biologies, Slough, UK) were amplified by PCR with primers containing restriction sites NcoI and NotI and subsequently inserted into pUC-HAV20 [14]. Sequences of the primers used are 5′-CCATGGCTCAGCCCCGAGAGCCACAGGTGTAC-3′ (forward, γ1/4), 5′-GCGGCCGCTCATTTACCCGGGGACAGGGAGAG-3′ (reverse, γ1), and 5′-GCGGCCGCTCATTTACCCAGAGACAGGGAGAG-3′ (reverse, γ4). The CH3 domains with leader sequence were cloned into a pcDNA3.1 expression vector (Invitrogen), obtaining respectively a pCDNA3.1-CH3(G1) and a pCDNA3.1-CH3(G4) vector. The CH3 domains were expressed by transfecting pCDNA3.1-CH3(G1) or pCDNA3.1-CH3(G4) expressing vector in HEK-293F cells using 293fectin and FreeStyleTM 293 expression Medium according to