A small domain (6.5 kDa) of bacterial protein G inhibits C3 covalent binding to the Fc region of IgG immune complexes

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Attachment of the complement component C3 to antigen-antibody (Ag-Ab) complexes (immune complexes, IC) is the key molecular event responsible for the elimination of many Ag in the form of Ag-Ab-C3b. The CH1 domain and the Fc region of the Ab, which have previously been involved in the binding of C3b, are also the targets of several bacterial IgG-binding proteins, particularly proteins G and A. Here we describe the ability of a small recombinant protein G domain (B2; 6.5 kDa) to inhibit the covalent binding of C3b to the Fc portion of IgG without affecting the binding to the Fab part. Protein G (B2 domain) produced a remarkable inhibition of covalent binding of C3b to IC formed with rabbit IgG, but none with the $F(ab')_2$ fragment, indicating that B2 interferes with the C3b binding to the Fc region. A weak inhibition was observed with IC formed with mouse IgG2b which preferentially binds B2 domain on the CH1 domain of the Fab. To confirm these data, recombinant single-chain Ab devoid of CH1 domains (scAb), and including the rabbit or human Fc portion (hinge-CH2-CH3), were produced and used to form IC. Protein G-B2 domain inhibited C3b binding to IC formed with scAb of either human or rabbit constant regions, supporting the view of a specific blockade of C3b binding to the Fc region. A similar inhibition of C3b binding was observed using protein A instead of protein G B2 domain and the same set of IC. On the CH1 domain, C3b and B2 bind on opposite faces, and therefore do not interfere with each other in their binding. However, B2 domain bound to the inter-CH2-CH3 region impedes the C3b binding to the Fc. This inhibition clarifies the specificity of C3b for the different regions of IgG and explains how bacterial IgG-binding proteins provide the bacteria with a mechanism of evasion from the opsonizing action of complement and contribute to the virulence. This could be a general mechanism of escape because protein G binds the majority of mammalian Ig.

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1 Introduction

One of the key events in the elimination of Ag or Ag-Ab complexes (immune complexes, IC) is the covalent binding of C3b to them. This binding promotes the solubilization and uptake of IC-C3b by mononuclear phagocytic cells bearing C3 receptors [1]. In recent years it has been shown that covalent binding of C3 is not only involved in Ag uptake, but also in later steps of Ag processing inside APC [2], which result in a higher efficiency of Ag presentation [3] and modulation of cell-mediated immune responses [4, 5]. To understand the molecular basis of these phenomena, several groups have studied the covalent complexes C3b-IgG and C3b-Ag [6–10]. The two C3b binding regions of IgG previously described (in the CH1 domain [6] and the Fc portion [7]) coincide with the two binding regions of some bacterial proteins (proteins A and G) on the Ab [11–13]. Hence we have studied whether a single domain of protein G (the B2 domain) could interfere with the covalent binding of C3b to individual domains of IgG. In addition, the use of recombinant Ab devoid of CH1 (scAb) allowed to discriminate the binding of C3b to the Fc or Fab region of the Ab. It was shown that the B2 domain of protein G directly interferes

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Abbreviations: **scFv:** VH and VL domains joined by a linker **C3α65, C3α43:** Fragments of C3 degradation **scAb:** Single-chain antibody of structure scFv-hinge-CH2-CH3 **IC:** Immune complexes **h:** Human **r:** Rabbit

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with C3b binding to the Fc region. A similar effect was observed with protein A since it binds in a region overlapping with that of protein G [12]. However, as B2 domain recognizes, on the CH1 domain, the face opposite to that which interacts with C3b it does not affect the C3b binding to the Fab. These data define the nature and specificity of C3b-IgG interaction, and explain how the inhibition of C3b binding endows pathogenic bacteria bearing IgG-binding proteins on their surface with a mechanism of escape from the action of complement.

2 Results

2.1 Structure and characterization of recombinant protein G B2 domain

Purified B2 domain produced a single band of about 6.5 kDa on SDS-PAGE. The amino acid composition and circular dichroism spectrum of recombinant B2 corresponded with that of the native protein.

2.2 Binding of protein G B2 domain to IgG, Fab and Fc fragments and scAb

Fig. 1 shows the interaction of recombinant protein G B2 domain with human, rabbit and mouse IgG and their respective Fab and Fc fragments. It reacted with mouse IgG2b and its Fab portion but not with the Fc region. In contrast the Fc fragment of rabbit and human IgG were the preferred binding region, although a weak interaction could be detected with the Fab of either IgG or the rabbit $F(ab')_2$ fragment. Both human (h)-scAb and rabbit (r)-scAb bound B2 domain, indicating the presence of a correctly folded Fc region, which maintains its binding properties. All the V regions used (the gene segments were sequenced; [8]) were the same (from the anti-HSA mAb). They belong to subgroups VH II and Vκ III which do not bind protein A [14]. Protein A interacted with both scAb, with mouse IgG2b and with human and rabbit IgG and its Fc regions. However, it did not bind to any of the Fab or $F(ab')_2$ fragments (not shown).

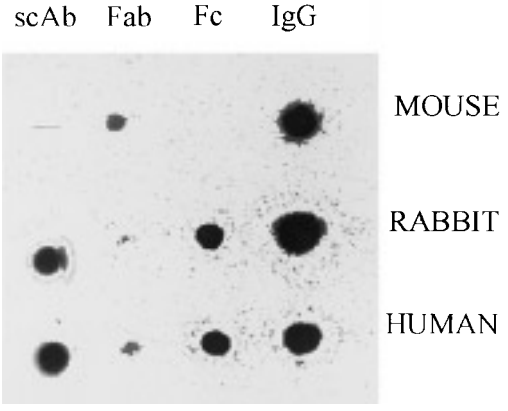

Figure 1. Detection on nitrocellulose filter of ^{125}I-labeled protein G B2 domain binding to scAb, IgG, Fab and Fc fragments from different sources.

2.3 B2 domain of protein G inhibits C3b binding to the Fc region of rabbit IgG

C3b binding to rabbit IgG-IC generates C3b-C3b-IgG covalent complexes which are detected on SDS-PAGE by the appearance of two major high molecular mass bands (C3α65-H chain, designated B and C3α65-C3α43, designated A) [7, 9] (Fig. 2A, lane 1). When the IC had been previously incubated with protein G (domain B2) (Fig. 2A, lane 2), a decrease in the intensity of both bands was observed. This effect was more evident in band B (C3α65-H chain). The inhibition is not complete and some IgG-C3b-C3b complexes should have been formed as indicated by the presence of band A. No high molecular mass bands are detected in the IC control incubated with EDTA (Fig. 2A, lane 3), indicating the specificity of the assay.

In the fluorography of the gel a decrease of about 87 % was estimated in the intensity of band B and a 69 % in band A (Fig. 2B, lane 2). Considering that protein G interacts preferentially with the Fc region of rabbit IgG (Fig. 1), the inhibition observed in Fig. 2B suggested that protein G would be interfering with the C3b binding to the Fc region of IgG. Hence similar experiments were performed using IC formed with the $F(ab')_2$ portion of rabbit IgG (Fig. 2C). The composition of bands A and B from $F(ab')_2$-IC has been previously characterized [15]; no changes could be observed in the band pattern of C3b binding to $F(ab')_2$-IC with (Fig. 2C, lane 2) or without (Fig. 2C, lane 1) protein G, indicating that it does not interfere with the covalent binding of C3b. This can be seen more clearly in the fluorography of the gel (Fig. 2D). The intensity of bands A and B is virtually identical in lane 1 (without protein G) and in lane 2 (with protein G) (9 % and 1 % less intensity for bands B and A, respectively), indicating that B2 domain does not interfere with the C3b binding to $F(ab')_2$-IC.

2.4 Protein G B2 domain inhibits C3b binding to scAb lacking the CH1 domain

C3b binding to scAb-IC generates, as shown by SDS-PAGE analysis, two high molecular mass bands, equivalent to the bands A and B described with the complete

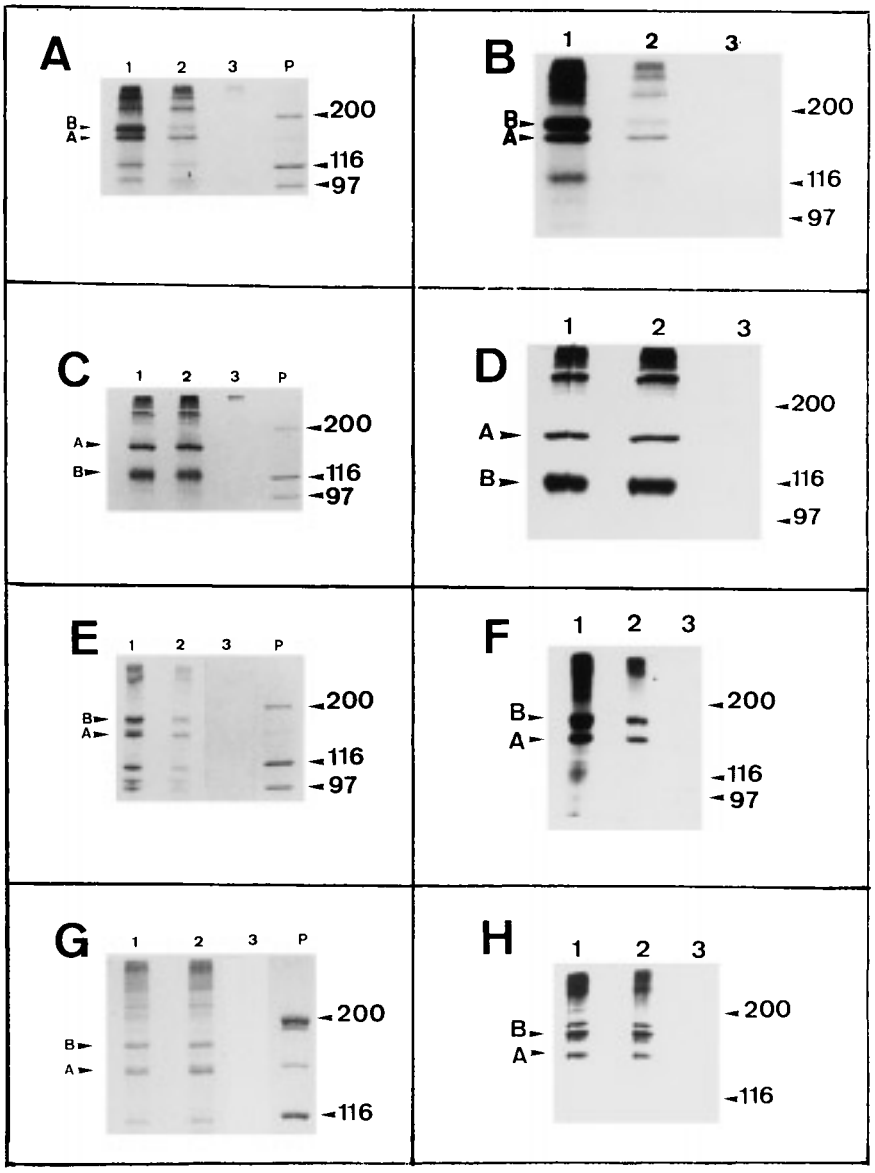

Figure 2. SDS-PAGE analysis, under reducing conditions, of C3b binding to IC. (A, C, E, G) stained gels; (B, D, F, H) the corresponding fluorographies. Lanes 1 and 2, assays in the absence or presence of B2 domain, respectively. Lane 3, control IC incubated with NHS-EDTA. Markers (in kDa) are shown on the right. (A/B) Rabbit IgG-IC; bands A (C3α65-C3α43) and B (C3α65-H) are named according to [9]. (C/D) Rabbit F(ab')$_2$-IC; the molecular composition of bands A and B has been described [15]. Note that in this case the M_r of band A is higher that of band B. (E/F) Human scAb-IC; bands B (C3α65-scAb) and A (C3α65-C3α43) appear at about 180 kDa and 160 kDa [8]. (G/H) Mouse mAb IgG2b-IC.

Ab (C3α65-C3α43 and C3α65-scAb; Fig. 2E, lane 1) [8]. In the presence of B2 domain a decrease in the intensity of both bands was observed (Fig. 2E, lane 2), similar to that described with rabbit IgG. The fluorography of this gel (Fig. 2F) shows that both bands are radioactive, indicating the participation of C3b (iC3b) in both of them (Fig. 2F, lane 1). In the presence of B2 domain, bands A and B decrease by 72 % and 65 %, respectively (Fig. 2F, lane 2), similar to the decrease observed with the complete antibody (Fig. 2A/B). In the scAb only the Fc region is available for protein G binding, so the inhibition observed should correspond to that of the Fc region, supporting the data obtained with the rabbit IgG-IC.

2.5 C3b binds to mouse IgG2b in the presence of B2 domain

In the light of the above data it seemed interesting to know whether C3b binding to mouse IgG2b could be inhibited by B2 domain, considering that it preferentially binds to the CH1 domain of the Fab (Fig. 1) [13, 16]. It can be observed in Fig. 2G that the intensity of bands A and B (Fig. 2G, lane 1) is only slightly affected by the previous incubation with B2 domain (Fig. 2G, lane 2). The fluorography (Fig. 2H, lanes 1 and 2) allowed to estimate a decrease of 25 % and 36 % for bands A and B, respectively. Hence B2 domain which has a high affinity for the CH1 domain, but shows a weak interaction with the Fc portion, produces only a modest inhibition of C3b binding to mouse IgG2b.

2.6 Protein A inhibits covalent binding of C3b to rabbit IgG-IC and scAb-IC

Experiments similar to that described with the B2 domain of protein G were carried out with protein A (42 kDa) and IC made with rabbit IgG, $F(ab')_2$, mouse IgG2b and the scAb (with human or rabbit Fc region). In every case the results paralleled those obtained with protein G, *i.e.* inhibition of C3b binding to rabbit IgG (70 % and 61 % for bands A and B, respectively) and scAb (82 % and 65 % for bands A and B), no effect on the $F(ab')_2$ fragment and a moderate inhibition with mouse IgG2b (not shown). The lack of inhibition with the $F(ab')_2$ fragment of rabbit IgG is obviously due to the absence of binding of protein A to this region of the Ab (see Fig. 1).

2.7 Protein G B2 domain does not produce solubilization of IC

Protein G B2 domain did not produce solubilization with any of the different IC tested in a wide range of Ag:Ab ratios [17]. The amount of radioactivity associated with the precipitated IC was always higher than 93 % of the original ^{125}I present in the labeled Ag and maintained in the precipitated IC after 12 h of incubation at 37 °C in the presence of B2 domain (data not shown).

3 Discussion

As a new approach to understand the C3b-IgG interaction we have looked for specific ligands which could inhibit this interaction. Bacterial proteins A and G are the best examples of proteins whose binding regions on the IgG molecule are fully characterized by X-ray diffraction ([11–13, 16]; Fig. 3). A relevant finding of this work was

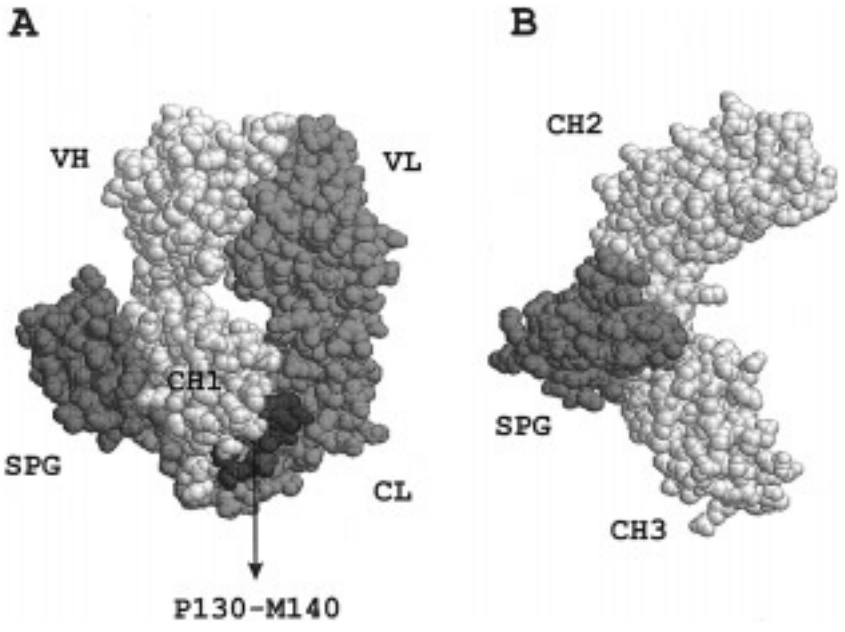

Figure 3. (A) Three-dimensional structure of protein G B2 domain (SPG)-Fab complex [13]. The C3b binding site on the CH1 [6] is marked (Pro 130-Met 140). This stretch is homologous between mouse and human IgG1. (B) Structure of protein G B1 domain-Fc complex; note that SPG denotes the B1 domain of protein G which is very similar structurally to the B2 domain [25].

that protein G did not inhibit C3b binding to IC formed with the F(ab')$_2$ fragment of rabbit IgG, but it did with the complete Ab (Fig. 2C/D and 2A/B, respectively). These data strongly suggest that the B2 domain inhibits C3b binding to the Fc region of the Ab, but it does not interfere with the binding to the CH1 domain. The latter was strongly supported by the results with mouse IgG2b: in spite of the strong binding of B2 to the CH1 domain [13], only a weak inhibition of C3b binding was observed (Fig. 2G/H), most probably due to binding of C3b on the Fc portion. It is apparent in Fig. 3 that the C3b and B2 binding regions (residues 125–147 and 209–216, respectively) lie on opposite faces of the CH1 domain, and therefore bound B2 domain does not interfere with C3b binding, supporting the lack of inhibition observed.

With rabbit IgG-IC, band B (C3α65-H) decreased more intensively than band A (C3α65-C3α43) (Fig. 2A/B), suggesting that B2 domain is inhibiting the binding of C3b to the H chain of Ab and not the C3b-C3b formation of those C3b molecules which have been able to attach to the Ab. Owing to its small size (6.5 kDa), B2 is covering a reduced area of the inter Cγ2-Cγ3 region on the Fc (50 kDa), indicating a discrete region of the Ab for C3 binding (Fig. 3). However, it cannot be ruled out that the inhibition of C3b binding to the Fc portion of IgG by the B2 domain is caused by a mere steric hindrance. When scAb-IC are tested for covalent binding of C3b in the presence of protein G B2 domain, the inhibition observed (Fig. 2E/F) is similar to that previously seen with the complete rabbit IgG (Fig. 2A/B). These data reinforce the view that the B2 domain can specifically inhibit C3b binding to the Fc region.

In vivo, binding of bacterial protein G or A to circulating Ig is likely to weaken the host's immune response and to facilitate infection, but the precise function of these proteins is still unknown. The inhibition of C3b binding to IgG should both provide selective advantages to bacteria and help to explain how complement activation and opsonization on bacterial surfaces is inhibited, contributing to the virulence of these pathogens. Presumably, *in vivo,* the inhibition of C3b binding to IgG by the complete protein G would be stronger than that observed *in vitro* with the small B2 domain. In this regard the inhibition of C3b binding by protein G is similar to that very recently described for protein H of *Streptococcus pyogenes* [18]. It is surprising how intracellular pathogens, such as mycobacteria, use C3b to invade macrophages [19], whereas extracellular bacteria (*Streptococcus* or *Staphylococcus*), bearing protein G or A, avoid the action of complement, preventing the binding of C3b.

In summary, we have shown that a small (6.5 kDa) domain from bacterial protein G which recognizes discrete areas in the Ab molecule is able to partially block the accessibility of C3b to IgG IC. The inhibition is, to great extent, restricted to the Fc region of the Ab. A similar inhibitory effect was observed using protein A. The inhibition of C3b binding to IgG bound on the surface of microorganisms through these bacterial IgG-binding proteins adds a new mechanism of evasion against the opsonizing action of complement.

4 Materials and methods

4.1 Expression, purification and labeling of protein G B2 domain

The expression vector pUC18-III containing the cloned B2 domain of protein G was kindly donated by Dr. J. Derrick, Manchester University, GB. *E. coli* (JM101 strain) was transformed with pUC18-III plasmid using the standard $CaCl_2$ method. The process of B2 purification has been described [20]. Labeling of B2 protein with ^{125}I was performed by the chloramine T method [21]. Purity and conformational integrity of protein G B2 domain was assesed by amino acids, SDS-PAGE and circular dichroism [22]. Protein A from *Staphylococcus aureus* (42 kDa; Pharmacia) was used without further purification.

4.2 Dot blot

IgG and its Fab or Fc fragments of human, rabbit or mouse origin, or the recombinant r-scAb or h-scAb were applied to nitrocellulose membranes and incubated for 3 h with ^{125}I-B2 domain (2×10^5 cpm/ml) according to [23]. After drying they were autoradiographed at –70 °C by using X-Omat films (KodaK). HSA was used as control.

4.3 Polyclonal and recombinant single-chain antibodies

The production and purification of anti-OVA polyclonal rabbit IgG and anti-HSA mouse mAb (LGF1/4.3.B9 hybridoma cell line) have been previously described [7–9]. Recombinant scAb were produced and expressed in SP2/0 murine myeloma cells. The scAb are composed of the V regions (VH and VL) of a mouse anti-HSA mAb covalently joined through a short linker, while the C-terminal VL domain was linked to the N-terminal domain of the human or rabbit Fc region through the hinge segment [8].

4.4 Ag-Ab aggregates

Two types of Ag-Ab aggregates (IC) were prepared. The first consisted of polyclonal rabbit IgG or its F(ab')$_2$ fragment anti-OVA and OVA as Ag [7, 9]. The second IC type was prepared with HSA as Ag and a mouse mAb IgG2b anti-HSA or

recombinant scAb bearing the same V region of the mAb and the constant portion (hinge-CH2-CH3) from human IgG1 (h-scAb) or rabbit IgG (r-scAb) [8].

4.5 SDS-PAGE and fluorography

SDS-PAGE and fluorography were carried out as previously described [7, 8]. Quantitation of band intensity was performed using the data from digitized fluorographies (Phosphoroimager; Molecular Dynamics) and the Image Quant software. Percent inhibition was calculated for bands A and B with rabbit IgG-IC taken as 100 % [9] (see below).

4.6 C3b binding assays

C3b binding assays under conditions which only allow the activation of the alternative pathway of complement have been described in detail [7–9]. IC incubated with human serum in the presence of EDTA (NHS-EDTA), which block both the alternative and classical pathways, were included as control. Inhibition assays of C3b binding to IC by B2 domain were performed (in quadruplicate) incubating IC with an excess of protein G (determined in previous experiments), during 12 h at 37 °C in PBS, pH 7.2. IC bearing B2 domain were washed and subjected to C3b binding assays in the presence of iodo-[^{14}C]-acetamide to detect the binding of C3b to the IC [7–9]. Finally they were centrifuged, washed and resuspended in sample buffer for SDS-PAGE. Similar experiments were performed with protein A. IC control incubated only with protein G B2 domain or protein A under the same conditions were included in every set of assays and analyzed by SDS-PAGE to verify the presence of the bacterial protein on the IC.

4.7 Solubilization of IC

Solubilization of IC containing ^{125}I-HSA or ^{125}I-OVA was performed according to [24]. Labeling of HSA and OVA was performed by the chloramine T method [21]. Different molar ratios of B2 domain : IC were tested, from 1 : 1 to 1 : 10 and for variable periods up to 24 h.

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