Characterisation of epitopes of pan-IgG/anti-G3m(u) and anti-Fc monoclonal antibodies

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Abstract

The characterisation of monoclonal antibodies (MAbs) and their epitopes is important prior to their application as molecular probes. In this study, Western blotting using IgG1 Fc and pFc' fragments was employed to screen seven MAbs before pepscan analysis to determine their reactivity to potentially linear epitopes. MAbs PNF69C, PNF110A, X1A11 and MAbs WC2, G7C, JD312, 1A1 detected epitopes within the C_H3 and C_H2 domains, respectively. However, only four MAbs showed pepscan profiles that highlighted likely target residues. In particular, MAbs PNF69C and PNF110A that have previously been characterised with pan-IgG and anti-G3m(u) specificity, detected the peptide motif 338-KAKGQPR-344 which was located within the N-terminal region of the C_H3 domain. Furthermore the majority of residues were present in all four IgG subclasses. Consequently the peptide identified was consistent with the pan-IgG nature of these antibodies. By using PCImdad, a molecular display programme, this sequence was visualised as surface accessible, located in the C_H2/C_H3 inter-domain region and proximal to the residue arginine₄₃₅. It is speculated that this residue may be important for phenotypic expression of G3m(u) and specificity of these reagents. Pepscan analysis of MAbs G7C and JD312 (both pan-IgG) highlighted the core peptide sequence 290-KPREE-294, which was present in the C_H2 domain and was common to all four IgG subclasses. PCImdad also showed this region to be highly accessible and was consistent with previous bioinformatic and autoimmune analysis of IgG. Overall these MAbs may serve as useful anti-IgG or anti-G3m(u) reagents and probes of immunoglobulin structure.

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1. Introduction

Assay restriction is a common feature and problem of monoclonal antibodies (MAbs). In essence, MAbs may perform well in some assay systems but may be poor or ineffective in others [1,2]. In addition, the specificity of a given reagent can also be determined by the assay

system employed [3]. This phenomenon relates to the nature and display of the antigenic determinant, or epitope, and whether it is modified (or denatured) as a result of procedures used within an assay technique [3]. Consequently the characteristics of an antibody's target epitope in terms of location, composition of amino acids and accessibility are highly significant together with issues of antibody specificity and reactivity in the development of secure assay systems [4]. To this end, pepscan methodologies which employ a series of overlapping synthetic peptides of a given protein have

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provided an effective means of identifying key residues for antibody recognition and binding [5]. Overall, pepscan analysis employing peptides immobilised on polyethylene pins has been satisfactorily used to identify MAb reactivity to continuous epitopes [6]. However, a disadvantage of this particular methodology has been the uncertainty of peptide regeneration and thus pepscan using biotinylated peptides (attached to streptavidin coated plates) has also been employed [7]. In general, pepscan methodologies provide greater resolution of target residues compared with gross epitope mapping techniques such as enzyme degradation and cyanogen bromide cleavage, which allow the localisation of antibody reactivity to particular fragments [1].

Monoclonal reagents are widely used within the biomedical sciences and readily enable the standardisation of both reagent and assay technique because they are specific and can be generated in unlimited quantities *in vitro* [8]. Earlier studies [9,10] have demonstrated two MAbs (PNF69C and PNF110A) with assay restriction profiles. Of interest is that both MAbs exhibit pan-IgG and anti-G3m(u) specificity in indirect ELISA/haemagglutination (HA) and capture ELISA/haemagglutination-inhibition (HAI), respectively. Both MAbs were derived from the same fusion but originated from different hybridoma cell lines. Both reagents may prove useful pan-IgG or anti-allotypic antibodies e.g. for clinical/forensic serology [11], although the target epitope(s) recognised by these antibodies remains unclear.

A previous report [12] has successfully employed biotinylated 15 mer overlapping peptides of IgG Fc to identify the target epitope of an in-house and commercially available pan-IgG monoclonal reagent, MAb A57H. This antibody recognised a peptide sequence 383-SNGQPENN-390 (Eu numbering, single amino acid code) that was found on all four IgG subclasses and straddled the fy1 strand and the b3 loop of the $\text{C}_\text{H}3$ domain [13]. As an extension to this work, we have used these peptides to map potential epitopes of MAbs PNF69C and PNF110A and five other anti-IgG Fc MAbs (X1A11, WC2, G7C, JD312, 1A1) previously characterised with regard to subclass specificity. Furthermore by employing a software programme 'PCImdad' we have been able to demonstrate the surface topography and accessibility of potential IgG target epitope's identified through pepscan analysis.

2. Materials and methods

2.1. Monoclonal antibodies

The generation and specificity of MAbs used in this study (PNF69C, PNF110A, X1A11, WC2, G7C, JD312 and 1A1) with regard to IgG subclass, allotypic (Gm) markers, deleted paraproteins and IgG fragments have

previously been reported [9,14–16]. These reagents have been characterised using a combination of techniques including haemagglutination (HA), haemagglutination-inhibition (HAI) and enzyme linked immunosorbent assay (ELISA). Five MAbs (TM15, ZB8, GOM1, ZG4 and RJ4) with reactivity to regions other than IgG1 Fc were used as controls and represented the predominant mouse isotype under investigation. Ascites (1 mg/ml) was used as a source of monoclonal reagent for all investigations.

2.2. Western blot analysis

Western blotting of MAbs was performed using standard procedures [1] on previously characterised paraproteins: PER (IgG1 Fc), pFc' PER (pepsin digestion product of Fc PER) [2,12–14] to ascertain reactivity to linear antigenic determinants. In brief, equal amounts (5 μg) of immunoglobulin preparations either containing 2-mercaptoethanol (denatured) or without reducing agent, were separated on SDS-12% polyacrylamide Laemmli gels. Proteins were then transferred onto nitrocellulose membrane (Hybond-C: Amersham Pharmacia Biotech Ltd., Little Chalfont, UK) and membranes blocked using 5% milk powder (Marvel) in PBS/Tween (0.1%). MAbs were used at a concentration of 30 $\mu\text{g}/\text{ml}$ diluted in PBS/Tween (0.1%). An anti-mouse horseradish peroxidase-conjugated secondary antibody (DAKO, UK) was used for the detection of transferred bands in conjunction with the enhanced chemiluminescence system (ECL, Amersham-Pharmacia Biotech UK Ltd.). The standard immunodetection protocol as detailed in the ECL handbook was used and the exposure time was 15 s.

2.3. Synthetic peptides

Ninety-four 15 mer peptides were provided by Mimotopes Pty Ltd., based on the human IgG1 Fc region, sequence NIE [17] and biotinylated at the N-terminus. Peptides were initially reconstituted in 200 μl DMSO and subsequently diluted in phosphate buffer diluent (see below) to give a final concentration of 1 nmol/ml. Peptides spanned most of the $\text{C}_\text{H}2$ and $\text{C}_\text{H}3$ domains: commencing 238-PSVFLFPPKPKDTLM-252 and terminating 424-FSCSVMEALHNHYT-438. All residues were related to the Eu numbering index [17] and the offset for overlapping peptides was two e.g. SRDELTKNQVSLTCL (354–368), DELTKNQVSLTCLVK (356–370). Sequences NIE and EU are virtually identical [17] although NIE possesses an additional residue: aspartic acid₃₈₉. In addition, EU and NIE exhibit amino acid replacements of glutamic acid₂₉₄ with glutamine₂₉₄, respectively.

2.4. Pepscan assay

Flat bottomed plastic microtitre plates (Nunc: Life Technologies, Paisley, UK) were coated with 100 µl of a 5 µg/ml solution of streptavidin (Sigma, Poole, UK) and left uncovered at 37 °C for at least 16 h until they had evaporated to dryness. The plates were washed using tapwater and each well blocked with 200 µl of a 2% solution of BSA in 0.1 M PBS pH 7.2. Plates were incubated for 1 h at room temperature, with agitation, and washed (x4) in PBS/Tween 20 (0.1% v/v). Peptides were prepared in diluent: PBS containing Tween 20 (0.1% v/v) and sodium azide (0.1% w/v), and 100 µl added to each well (ca. 30 pmol of biotinylated peptide per well). Plates were incubated for 1 h at room temperature, with agitation, and then washed (x4) in PBS/Tween. MAb ascites were diluted 1/1000 in diluent and added to wells (100 µl). Plates were incubated for 1 h at room temperature with agitation and then washed (x4). Finally peroxidase-conjugated goat anti-mouse immunoglobulin (DAKO, Cambridge, U.K.) was optimally diluted (1/1000) in 0.01 M PBS pH 7.2 containing 1% v/v sheep serum, 0.1% v/v Tween 20, 0.1% w/v sodium caseinate and applied to wells (100 µl/well): plates were incubated for 1 h at room temperature with agitation. Following washing, 100 µl of ABTS (Sigma, Poole, UK) substrate (dissolved in 0.1 M di-sodium hydrogen orthophosphate, 0.08 M citric acid) containing 0.01% w/v H₂O₂ was added to each well. The colour reaction was allowed to proceed for 30 min and the OD read at 405 nm using a Titertek Multiscan MC microplate reader. OD values were provided as 0–2000 nominal OD Units (equivalent to the generally accepted format 0.0–2.0 OD Units) [1–3]. The OD values

obtained from control MABs were used to determine a mean value for each peptide that was subtracted from all other Pepscan profiles (for controls: range, 94–1071 OD Units; mean, 230 OD Units, maximum OD [peptide 62, 360-KNQVSLTCLVKGFYP-374, followed by peptide 61, 358-LTKNQVSLTCLVKGF-372]).

2.5. PCImdad display

A PC interactive molecular display and design programme (PCImdad) [18] was used to display potential amino acids on the Fc region of IgG derived from Pepscan data. Key residues were highlighted and displayed, employing space-filling and carbon backbone modes, on an IgG Fc molecule [19].

3. Results

3.1. Western blotting

To ascertain the reactivity to potential linear epitopes within IgG Fc, seven MABs (PNF69C, PNF110A, X1A11, WC2, G7C, JD312, 1A1) were evaluated by Western blotting. In this system, both reduced and non-reduced Fc and pFc' fragments were used and their electrophoretic transfer to nitrocellulose validated by previously characterised antibodies. The data (Table 1) highlighted that MABs PNF69C and PNF110A detected both reduced and non-reduced pFc' (PER). MABs X1A11 and 1A1 were reactive with Fc and pFc' fragments whilst MABs WC2, G7C and JD312 were reactive with Fc(PER), but not pFc' (PER).

3.2. Pepscan profiling

Out of seven antibodies tested, only four MABs (PNF69C, PNF110A, G7C and JD312) generated Pepscan profiles. MABs X1A11, WC2 and 1A11 showed similar reactivity to peptides as control antibodies. The Pepscan profile for MAB PNF69C (Fig. 1a) highlighted antibody activity for peptides 47–51 with OD's ranging from 1500 to 2000 OD units. Identical peptides were detected by MAB PNF110A (not shown) that on alignment suggested a core peptide sequence: 338-KAKGQPR-344. Both antibodies exhibited reactivity for peptides 61 (358-LTKNQVSLTCLVKGF-372) and 62 (360-KNQVSLTCLVKGFYP-374) that was also noted for all control MABs tested. MAB G7C identified peptides 23–26 (Fig. 2a) with antibody activity ranging from 1500 to 2000 OD units. Marginally higher activity (2000–2500 OD units) was also shown by MAB JD312 and included peptides 22–27. Alignment of peptides for MABs G7C and JD312 highlighted the core sequence: 290-KPREE-294.

Table 1
Reactivity of MABs in Western blotting using IgG1 Fc and pFc' fragments

Mab	Fc(PER)		pFc' (PER)	
	Non-red.	Red.	Non-red.	Red.
PNF69C	+	+	+	+w
PNF110A	+	+	+	+
X1A11	+	+	+	+
WC2	+	NT	–	NT
G7C	+	+	–	–
JD312	+	+	–	–
1A1	+	NT	+	NT
<i>Controls</i>				
A57H	+	+	+	+
A55	+	+	–	–

MABs were evaluated for reactivity in Western blotting against non-reduced (non-red.) and reduced (red.) paraproteins Fc(PER) (MW 50 KDa) [9,15] and the pepsin derived fragment pFc' (PER) (MWt 25 KDa) [9,15]. + (positive), – (negative), NT (not tested), w (weak band). Control MABs A55 and A57H detected C_H2 and C_H3 domains, respectively.

Fig. 1. (a) Pepscan analysis of MAb PNF69C. The profile for MAb PNF110A was similar and specific details are presented in text. (b) PCImdad of peptide sequence 338-KAKGQPR-344 plus the position (arrowed) of arginine₄₃₅.

3.3. PCImdad display

The location and surface topography of likely target epitopes was visualised on an IgG molecule using the space-filling mode. The core sequence 338-KAKGQPR-344 (Fig. 1b) was present on an exposed peptide segment (or loop) that bridged the fy3 face and the fx1 face of the $\text{C}_{\text{H}2}$ and $\text{C}_{\text{H}3}$ domains, respectively. Conversely the peptide sequence 290-KPREE-294 was located on the fx3 face of the $\text{C}_{\text{H}2}$ domain which was exposed and solvent accessible (Fig. 2b) and thus capable of interaction with antibody.

4. Discussion

Epitope mapping using pepscan provides a useful adjunct to the characterisation of MABs [4] and has been used in identifying epitopes on macromolecules including allergens and components of viruses [20,21]. However, an important caveat of pepscan is that the technique is biased towards antibodies recognising linear, as opposed to conformational epitopes. Petrakou et al., [22] showed that only half of the MABs investigated using overlapping peptides of MUC1 mucin yielded pepscan profiles. To circumvent this problem,

we initially tested our panel of MABs in Western blotting since the detergent sodium dodecyl sulphate effectively linearises proteins for gel separation prior to their transfer to nitrocellulose membrane [1]. In our study all seven MABs exhibited reactivity in Western blotting: MABs PNF69C, PNF110A, X1A11, 1A1 to pFc'(PER) and MABs WC2, G7C, JD312 to Fc(PER). Since pepsin cleaves C-terminal to glutamic acid₃₃₃ to yield a pFc' fragment [13], it may be inferred that MABs WC2, G7C and JD312 bound to epitopes within the $\text{C}_{\text{H}2}$ domain of Fc(PER). Whilst it has been recognised that some renaturation of proteins may occur following SDS leaching i.e. during electrophoretic transfer [23], it was likely that these antibodies bound to potentially linear Fc epitopes. Overall preliminary evaluation of antibodies in Western blotting confirmed previous $\text{C}_{\text{H}2}/\text{C}_{\text{H}3}$ localisation studies of anti-Fc MABs [9,15,16] but was only partially successful since MABs X1A11, WC2 and 1A1 did not yield pepscan profiles. In explanation, it was plausible that these antibodies recognised quasi-linear epitopes that were perhaps conformationally constrained by amino acids upstream/downstream of the target site. Furthermore, the weaker reactivity of MAB PNF69C for its epitope on reduced pFc' may also suggest some conformational constraints. Clearly the use of small isolated peptides attached to microtiter

Fig. 2. (a) Pepscan analysis of MAb G7C. The profile for MAb JD312 was similar and specific details are presented in text. (b) PCImdad of peptide sequence 290-KPREE-294.

plate wells may have compromised antibody–epitope interactions and it will be of interest to further evaluate the epitopes of MAbs X1A11, WC2 and 1A1 using site-directed mutagenesis [24] and phage display [25].

For MAbs PNF69C and PNF110A pepscan analysis highlighted the core peptide sequence 338-KAKGQPR-344 which was located at a position that included C_H2/C_H3 interdomain residues and the N-terminal region of the C_H3 domain. Furthermore this motif was present in all four IgG subclass proteins, although alanine₃₃₉ was substituted for threonine₃₃₉ in IgG2 and IgG3 allotypic variants (Table 2). A previous study [9] suggests that the pan-IgG reactivity of both MAbs was independent of this amino acid substitution. Thus pepscan of MAbs PNF69C and PNF110A highlights a common IgG subclass epitope that explains the pan-IgG nature of these reagents. In using PCImdad the peptide was characterised as highly accessible and, therefore, likely to be antigenic. Interestingly epitope mapping of polyclonal and monoclonal rheumatoid factors [26–28] highlights three C_H3 domain epitopes (343-PREPQVY-349, 346-PQVYTLP-352, 350-TLPPSRE-356) that either overlap or are immediately adjacent to the peptide motif 338-KAKGQPR-344. Taken together, these studies reveal a highly antigenic region targeted by autoimmune antibodies [28] and MAbs developed in

mice [9]. Evidently from previous studies using intact IgG [9,15,16] the glycan moiety does not appear to hinder the accessibility of MAbs PNF69C and PNF110A for their target epitopes.

The location of the G3m(u) allotypic marker has previously been suggested to reside within the C_H2 domain of IgG3 and its expression linked to the presence of threonine₃₃₉ (C_H2 domain) and possibly arginine₄₃₅ in the C_H3 domain [29,30]. However, current sequencing information [17] does not support a role for threonine₃₃₉ since Table 2 reveals that G3m(u) paraproteins WI and OM contain threonine₃₃₉ and alanine₃₃₉, respectively, whilst paraprotein JIR G3m(st) possesses threonine₃₃₉. This observation is also confirmed by Dard et al. [31] who investigated IgG3 allotypes in human populations other than G3m(u). In our studies it was also apparent that alanine₃₃₉ was present within the peptide sequence 338-KAKGQPR-344. Overall these observations suggest that the presence alanine/threonine₃₃₉ may be less significant in the phenotypic expression of G3m(u). Consequently this allotypic marker may be conformationally dependent and necessitate one or more additional residues on IgG3. Intriguingly Table 2 shows that IgG3 variants expressing G3m(gu) and G3m(bu) possess arginine₄₃₅ whilst IgG1, IgG2, IgG4 and G3m(st) paraproteins

Table 2

Location of motifs identified through pepscan within IgG subclasses and Gm allotypic variants

	Eu index	IgG1		IgG2	IgG3			IgG4	
		Eu(f)	Cl(za)	Cl(n)	WI(bu)	OM(gu)	^a JIR(st)	Cl(n)	G4m(a)
MAb: G7C JD312	290	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Motif: K
	291	Pro	Pro	Pro	Pro	<i>Leu</i>	Pro	Pro	P
	292	Arg	Arg	Arg	Arg	Arg	Arg	Arg	R
	293	Glu	Glu	Glu	Glu	Glu	Glu	Glu	E
	294	Gln	Glu	Glu	Glu	Glu	Gln	Glu	E
Mab: PNF69C PNF110A	338	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Motif: K
	339	Ala	Ala	<i>Thr</i>	<i>Thr</i>	<i>Ala</i>	<i>Thr</i>	Ala	A
	340	Lys	Lys	Lys	Lys	Lys	Lys	Lys	K
	341	Gly	Gly	Gly	Gly	Gly	Gly	Gly	G
	342	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Q
	343	Pro	Pro	Pro	Pro	Pro	Pro	Pro	P
	344	Arg	Arg	Arg	Arg	Arg	Arg	Arg	R
	435	His	His	His	<i>Arg</i>	<i>Arg</i>	His	His	

Key allotypic residues shown in italic: *Thr*₃₃₉ associated with G3m(u) [26,27] but also present in JIR G3m(st).

^a Note that paraprotein GOE G3m(st) possesses *Ala*₃₃₉ together with paraproteins OM G3m(gu). *Leu*₂₉₁ is associated with G3m(g) [10]. Core pepscan motifs shown in bold (single letter amino acid code: K, lysine; A, alanine; G, glycine; Q, glutamine; P, proline; R, arginine; E, glutamic acid). *Arg*₄₃₅, present in paraproteins expressing G3m(u) is underlined.

possess histidine₄₃₅. In using PCImdad, we show that residue 435 is surface accessible and spatially orientated adjacent to the peptide sequence 338-KAKGQPR-344. Consequently we speculate that the proximity of arginine₄₃₅ in conjunction with the peptide motif may play a role in the conformation and phenotypic expression of G3m(u). It is, therefore, conceivable that the manner of epitope display afforded by different assay systems could influence the 'dual' reactivity i.e. anti-G3m(u)/pan-IgG of MAbs PNF69C and PNF110A observed in previous studies [9,14]. In the latter, G3m(u) specificity was shown when IgG was captured in ELISA or coated onto red blood cells whereas pan-IgG reactivity was noted with IgG coated directly onto microtitre plates. In this case, hydrophobic interactions between immunoglobulin and a plastic surface could bias the reactivity towards a common IgG epitope as opposed to a potential conformation (G3m(u)) epitope.

Epitope mapping of MAbs G7C and JD312 identified the core peptide sequence 290-KPREE-294 that was present in all four IgG subclasses (Table 2) and was consistent with the pan-IgG reactivity exhibited by these antibodies in previous studies [15,16]. The latter also serve to demonstrate that the binding of MAbs G7C and JD312 to IgG is not rendered inaccessible by oligosaccharide moieties linked via asparagine₂₉₇ [32] or influenced by leucine₂₉₁. PCImdad also highlighted a surface topography for this motif that was highly exposed, solvent accessible and on a loop region (i.e. connecting two β -strands). These observations were consistent with bioinformatic tools [33] which have been used to predict regions of antigenicity on IgG1 Fc [34]: analysis shows the sequence 290-KPREQQYN-297 as extremely hydrophilic and thus a likely antigenic site within the C_H2

domain. Intriguingly pepscan analysis of rheumatoid factors [26–28] likewise highlight an epitope (274-KFNWYVD-280) adjacent to the peptide sequence 290-KPREE-294 suggesting a key C_H2 antigenic region.

Pepscan of all seven MAbs showed reactivity to peptide 62 (360-KNQVSLTCLVKGFYP-374) and to a lesser extent peptide 61 (358-AAQVSLTCLVKGF-372). Since this phenomenon was observed for all control antibodies, it was unlikely that these peptides represented a major epitope for MAbs PNF69C, PNF110A, G7C and JD312. It was possible that some reactivity could be attributed to non-specific interactions of peptide, MAb and secondary antibody. In terms of location, peptide 62 was situated on the b1 loop and proximal region of the fx2 face of the C_H3 domain [13]. Hence this peptide was remote to the C_H2/C_H3 domain interface and thus unlikely to contribute to epitope(s) recognised by MAbs PNF69C and PNF110A. Minor variations in pepscan profiles were also observed for MAb pairs PNF69C, PNF110A and G7C, JD312 that could reflect subtle differences in the spatial orientation of a MAb for a given epitope. Similar variations in reactivity have been reported in other studies [15,16]. Interestingly preliminary data from BIAcore analysis [35] using purified IgG paraprotein immobilised with a goat anti-mouse antibody, highlights differing affinity constants [PNF69C (4×10^7 per M), PNF110A (7.14×10^7 per M), G7C (2.70×10^8 per M), JD312 (2.86×10^8 per M), TM15 (2×10^8 per M)] that might reflect subtle variations in Mab–epitope interactions.

The present study has shown the combination of pepscan and surface topography visualisation as a means of localising and characterising epitopes of a panel of anti-IgG Fc monoclonal reagents. It is specu-

lated that the anti-G3m(u) specificity of MAbs PNF69C and PNF110A may be influenced by the presence of arginine₄₃₅ in proximity to the peptide motif expressed in all four IgG subclasses. Overall the MAbs investigated in this study may prove useful probes of antigenic structure e.g. for evaluating recombinant antibodies, or reagents for diagnostic/forensic purposes. In addition, they may serve in functional assays to inhibit the binding of neonatal FcRn (MAbs G7C, JD312) and Streptococcal protein G (MAbs PNF69C and PNF110A) [30].

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