Original Article

Production and Characterization of Mouse Monoclonal Antibodies Recognizing Human Pan-IgG Specific Conformational or Linear Epitopes

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Abstract

Background: Pan-IgG specific monoclonal antibodies (MAbs) are essential tools for assessment of humoral immunity, immune deficiency and gammopathy. In this study, four hybridoma clones producing MAbs with different specificities for human IgG isotypes were established.

Methods: Splenocytes from Balb/c mice immunized with Fc fractions of human IgG were fused with SP2/0 myeloma cells. Hybridoma cells were selected in HAT selective medium and cloned by limiting dilution assay. Antibody-secreting cells were screened by enzyme-linked immunosorbent assay (ELISA) and the specificity of secreted MAbs was further analyzed using a panel of purified myeloma IgG proteins by ELISA and immunoblotting. Cross-reactivity to immunoglobulins (Igs) of other species was studied by indirect ELISA using serum samples collected from 9 animals.

Results: Immunoblotting studies revealed recognition of either linear or conformational epitopes by these MAbs. The most abundant cross-reactivity (100%) was observed with monkey Igs, while no cross-reactivity was detected with rabbit, guinea pig, sheep, goat, cat and hen sera. Two of the MAbs cross-reacted with either dog or horse sera. The affinity constant of two MAbs was measured by ELISA and found to be 0.74×10^{8} M^{-1} and 0.96×10^{7} M^{-1}.

Conclusion: Our results indicate that these pan-IgG specific MAbs recognize restricted linear or conformational epitopes located on all human IgG subclasses with no cross-reactivity to IgG from most species studied. These MAbs are potentially useful tools for quantification of total or antigen-specific IgG levels.

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Introduction

Human immunoglobulins are broadly classified into five major serologically different heavy chain isotypes (IgM, IgG, IgA, IgD and IgE) and two distinct light chain isotypes (Kappa and Lambda) [(1)]. Each heavy chain isotype has different biological characteristics [(2)] and a particular profile of effector function [(3)]. Antibody isotypes are differently expressed in response to different antigenic stimuli [(4-6)]. Thus, different patterns of antigen specific Igs [(7,8)] have been reported in a variety of diseases.

IgG is the most abundant immunoglobulin present in human serum and extravascular fluids and there might be a relationship between the IgG level and severity of diseases [(9-11)]. Therefore, IgG has a great diagnostic value [(12)]. Furthermore, analysis of the IgG profiles

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to different antigens of a microorganism might reveal different profiles of T helper associated responses, [13] and hence, is helpful for designing a potential vaccine [14]. Quantification of total and antigen specific IgG levels depends on the availability of specific MAbs and sensitivity of assays could be increased with assay optimization, standardization [15] and application of high affinity MAbs. This is the advantage of MAbs in comparison to polyclonal anti IgG antibodies.

In the present study, four MAbs with specificity for different epitopes shared by all human IgG subclasses are produced and characterized.

Materials and Methods

Preparation of purified human IgG subclasses

A panel of 30 different purified human Ig myeloma proteins of different isotypes and IgG myeloma proteins of known IgG subclasses and light chain types was employed in this study. These myeloma proteins, obtained from patients with multiple myeloma, were either purified by diethyl aminoethyl (DEAE) cellulose (Whatmann, UK) chromatography or by affinity chromatography using Staphylococcal protein A (SPA) or Streptococcal protein G (SPG) Sepharose 4B (Pharmacia, Sweden). The heavy chain and light chain isotypes and subclasses of myeloma proteins were identified using specific mouse MAbs including: AF6 (IgM), 8a4 (IgG), 2D7 (IgA), JA11 (IgD), C4 (λ), 6el (κ), JL512 (IgG1), GOM2 (IgG2), ZG4 (IgG3) and RJ4 (IgG4), kindly provided by Professor R. Jefferis and Dr. M. Goodal (Dept. of Immunology, University of Birmingham, UK).

Polyclonal IgG were prepared as described previously [16]. Briefly, polyclonal IgG was isolated from normal serum with SPG-Sepharose column. Fc and Fab fragments were produced from several purified human myeloma proteins of the IgG isotypes, by papain digestion [17]. Digested fragments were isolated by affinity chromatography with SPG- or SPA-Sepharose column.

Animal sera

Sera from human and nine animals were prepared from their clotted blood. The animal species used in this study were chicken, rabbit, guinea pig, cat, dog, sheep, goat, horse and monkey. The human serum was used as a control.

Production and selection of hybridomas

Balb/c mice were used for hybridoma production as described else where [18]. Briefly, Balb/c mice (8-12 weeks of age) were immunized with four intraperitoneal injections of Fc fragments of IgG myeloma proteins emulsified in Freund´s complete adjuvant (Sigma, USA) (first injection) or incomplete adjuvant (Sigma, USA) (other injections) (50 μg, every 2 weeks). Three days after the last injection, spleen cells were fused with SP2/0 myeloma cells (NCBI 129, National Cell Bank of Iran, Pasteur Institute of Iran, Tehran), using polyethyleneglycol (PEG 1500) (Sigma, USA). Hybridomas were grown in DMEM culture medium (Sigma, USA) containing 20% Fetal Calf Serum (FCS) (Seromed, Germany), penicillin (100 *IU/ml*) and streptomycin (100 $\mu g/ml$) and supplemented with hypoxantine (1×10^{-4} *M*), aminopterin (4×10^{-7} *M*) and thymidine (1.6×10^{-5} *M*) (HAT) (Sigma, USA). Ten to 14 days after fusion, secreting hybrids were identified by analysis of culture supernatants by the ELISA technique described below. Selected antibody producing cultures were cloned by limiting dilution assay [19]. Clones secreting antibody of desired reactivity were expanded in 25 and 75 cm^2 flasks (Nunc, Denmark), harvested and cryopreserved in 40% FCS and 10% dimethylsulfoxide (DMSO) (Sigma, USA).

Analysis of specificity of MAbs by indirect ELISA

Specificity of MAbs was determined by ELISA technique as described elsewere [20]. Brifely, microtiter polystyrene plates (Maxisorp, Nunc and Denmark) were coated with 1-10 $\mu g/ml$ of purified myeloma IgG subclasses, including the immunogen and its fragments (Fab and Fc) or polyclonal IgG in PBS

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(0.15 *M*, pH=7.2). Then 0.05 *ml* of culture supernatant was added. Appropriate dilution of HRP-conjugated sheep antimouse Ig (prepared in our lab) was then added and the reaction revealed with O-phenylenediamine dihydrochloride (OPD) (Sigma, USA) substrate. Finally, the reaction was stopped with 20% H2SO4 and the Optical Density (OD) measured by a multiscan ELISA reader (Organon Teknika, Boxtel, Belgium) at 492 *nm*.

Isotype determination of MAbs by capture ELISA

Goat antimouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM at 1/1000 dilution, were adsorbed on to the wells of a microtitre ELISA plate (Nunc, Denmark). Isotypes of MAbs in culture supernatants were determined according to the ELISA technique mentioned above.

Affinity constant (K_{aff}) determination by ELISA

We determined the K_{aff} by ELISA technique as described elsewhere [21]. Brifely, ELISA plates (Nunc, Denmark) precoated with four different concentrations of human IgG ([Ag], [Ag′] and [Ag″]) were separately incubated with serial concentrations of each MAb. Sigmoid curves were constructed using the OD values obtained for different concentrations of each MAb. Four non-overlapping curves were selected for each MAb to calculate the affinity constant. The half maximum OD (OD-50) was assigned for all selected curves from which the corresponding antibody concentration ([Ab], [Ab′] and [Ab″]) was extrapolated. Accordingly, [Ab] and [Ab′] are the measurable total Ab concentrations at OD-50 and OD′-50 for plates coated with [Ag] and [Ag′], respectively. The affinity constant was determined using the following equation [22]:

$K_{aff} = (n-1)/2(n([Ab']t - ([Ab]t)$

Where n= [Ag]/[Ag′]

Immunoblotting technique for analysis of specificity of MAbs

Specificity of MAbs was assessed by immunoblotting technique as described elsewere [23]. Brifely, affinity purified myeloma IgG subclasses, polyclonal IgG and their fragments were electrophoresed under native and denaturing conditions in 10% polyacrylamide gel (SDS-PAGE) (Sigma) and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany). After blocking with 2.5% skim milk (Merck, Germany), the membrane was incubated with culture supernatants of MAbs for 1.5 *hr* at 37°*C*, followed by HRP-conjugated sheep anti-mouse Ig. The bands were finally visuallized with diaminobenzidine tetrahydrochloride (DAB) (Sigma) substrate.

Results

Screening and selection of IgG specific hybridomas

Culture supernatants from growing hybridomas were screened by ELISA using a panel of five Ig heavy chain isotypes and four IgG

Table 1. Reactivity of selected hybridoma clones with human Ig isotypes, polyclonal IgG and IgG subclasses

Screening antigen	OD (492 *nm*)								
Hybridoma	**IgM**	**IgA**	**IgD**	**IgE**	**Polyclonal IgG**	**IgG1**	**IgG2**	**IgG3**	**IgG4**
3F2D8	<0.1	<0.1	<0.1	<0.1	**1.7**	1.5	1.6	1.9	1.6
1F4C4	<0.1	<0.1	<0.1	<0.1	**1.5**	1.6	1.4	1.4	1
8F9A8	<0.1	<0.1	<0.1	<0.1	**1.6**	1.8	1.7	1.6	1.4
5F19G11	<0.1	<0.1	<0.1	<0.1	**1.1**	1.4	1	1.2	1.4
Anti IgG1(JL512)	<0.1	<0.1	<0.1	<0.1	**1.3**	**1.2**	0.27	0.3	0.27
Anti IgG2(GOM2)	<0.1	<0.1	<0.1	<0.1	**1.6**	0.4	**2**	0.3	0.25
Anti IgG3(ZG4)	<0.1	<0.1	<0.1	<0.1	**1.3**	0.3	0.3	**1.9**	0.3
Anti IgG4(RJ4)	<0.1	<0.1	<0.1	<0.1	**1.4**	0.3	0.3	0.3	**1.6**

* Positive reactivity is shown in bold figures. Commercial IgG subclass monospecific MAbs, including JL512 (IgG1), GOM2 (IgG2), ZG4 (IgG3) and RJ4 (IgG4), are included as control MAbs for each IgG subclass

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Table 2. Determination of the isotype of monoclonal antibodies by ELISA

Isotype MAbs	IgG1	IgG2a	IgG2b	IgG3	IgA	IgM
3F2D8	**0.8**	0.2	0.2	0.2	0.2	0.2
1F4C4	**0.9**	0.2	0.2	0.17	0.17	0.18
8F9A8	**0.7**	0.2	0.2	0.17	0.18	0.2
5F19G11	**0.65**	0.2	0.1	0.1	0.1	0.2
Control⁻	0.16	0.14	0.1	0.15	0.1	0.16

Control⁻ : Culture supernatant from SP2/0 myeloma cells. The results represent OD values obtained at 492 *nm*

these MAbs reacted only with Fc, but not Fab fragments of their immunogens (Figure 3).

Cross-reactivity studies employing whole sera from a range of animal species indicated the most abundant cross-reactivity (100%) with monkey Igs, while no cross-reactivity was detected with rabbit, guinea pig, sheep, goat, cat and hen serum. Two MAbs cross-reacted with either dog (3F2D8) or horse

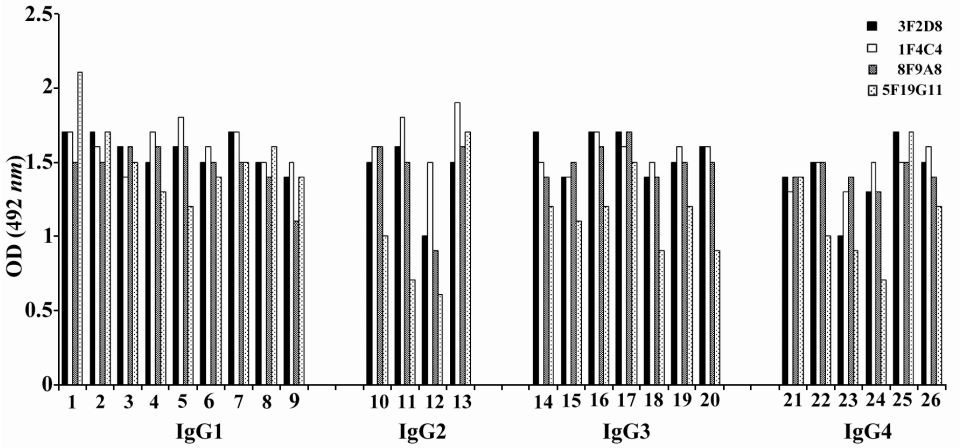

Figure 1. Reactivity patterns of pan-IgG specific monoclonal antibodies with human IgG subclasses

subclasses. Hybridomas secreting MAbs reactive with all IgG subclasses with no reactivity to IgM, IgD, IgA or IgE were selected. Results obtained for the selected hybridoma clones are illustrated in table 1.

Characterization of MAbs

Following cloning and subcloning, culture supernatants from the selected hybridomas were further characterized. All MAbs belonged to IgG1 isotype (Table 2). Specificity of these MAbs was determined, using a panel of purified myeloma proteins, including IgM, IgG, IgA, IgD, IgE, IgG1 (n=9), IgG2 (n=4), IgG3 (n=7) and IgG4 (n=6) subclasses.

According to ELISA and immunoblotting studies, all MAbs were pan-IgG specific (Figure 1). Three MAbs (3F2D8, 8F9A8 and 5F 19G11) recognize linear epitopes, while the remaining MAb (1F4C4) reacts with a conformational epitope located on heavy chain of all IgG subclasses. Representative immunoblotting results are shown in figure 2. All

Table 3. Cross-reactivity of monoclonal antibodies with animal sera

Animal sera	Cross-reactivity% *			
	3F2D8	1F4C4	8F9A8	5F19G11
Monkey	100	100	66	84
Rabbit	<0.1	<0.1	<0.1	<0.1
Guinea Pig	<0.1	<0.1	<0.1	<0.1
Sheep	<0.1	<0.1	<0.1	<0.1
Goat	<0.1	<0.1	<0.1	<0.1
Cat	<0.1	<0.1	<0.1	<0.1
Dog	**87**	<0.1	<0.1	<0.1
Horse	<0.1	**48.5**	<0.1	<0.1
Hen	<0.1	<0.1	<0.1	<0.1

*% Cross-reactivity is expressed relative to the value obtained for human pooled serum

(1F4C4) serum Igs (Table 3).

Determination of affinity of MAbs

The ascitic fluids of two MAbs (3F2D8 and 5F9G11) were prepared and subsequently purified by affinity chromatography using SPG column. The affinity constant (K_{aff}) of these two MAbs was determined by ELISA.

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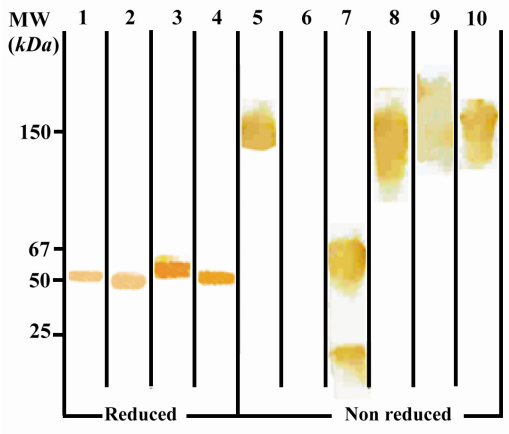

Figure 2. Immunoblot analysis of 3F2D8 monoclonal antibody reactivity with human IgG subclasses. Lanes 1 to 4 represent reduced forms of IgG1, IgG2, IgG3 and IgG4 paraproteins, respectively. Lanes 5 to 7 represent non reduced forms of whole molecule, Fab and Fc fragments of IgG1. Lanes 8 to 10 represent non reduced forms of IgG2, IgG3 and IgG4 paraproteins, respectively

Triple serial concentrations of the antigens and MAbs were selected to construct the corresponding curves and to extrapolate the K_{aff} values using the formula given in the Materials and Methods. Representative curves obtained for the MAbs are illustrated in figure 4 (A, B) and the calculated average K_{aff} values are presented in table 4.

Discussion

In this study, four MAbs specific for isotypic epitope(s) of human IgG were produced and characterized. These MAbs were specific for Fc-dependent isotypic epitopes common to all IgG subclasses. As shown by immunoblotting studies, three of these pan-IgG-Fc specific MAbs (3F2D8, 8F9A8 and 5F19G11)

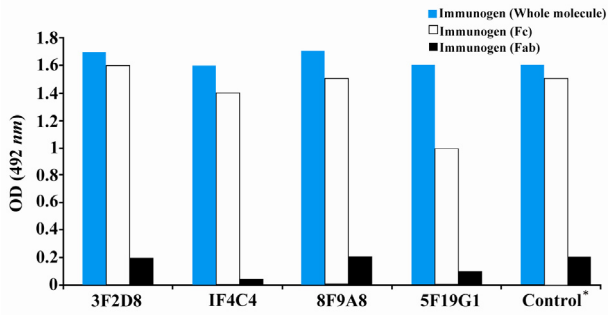

Figure 3. Reactivity of pan-IgG specific monoclonal antibodies with enzymatic fragments of IgG. Control represents a mouse monoclonal antibody (8a4) with anti human pan-IgG specificity

reacted with linear epitope(s) and one MAb (1F4C4) reacted with a conformational epitope located to all human IgG subclasses.

Several MAbs specific for a variety of epitopes located to human IgG heavy chain have so far been produced by other investigators [24-27]. A few anti IgG MAbs were shown to be IgG-Fab specific [25], whereas most IgG-specific MAbs were found to recognize Fc-associated epitopes, a specificity similar to all of our MAbs (Figure 3). This is in agreement with another study [26] in which a large number of MAbs were produced with specificity for conformational epitopes located on the CH2-CH3 border of the hinge region, indicating the high immunogenicity of this region. Those MAbs are very similar to one of our MAb (1F4C4) which recognizes a conformational epitope located to the Fc region of IgG molecules. Nevertheless, three of our MAbs recognized linear epitopes.

The four MAbs produced in this study

Table 4. Affinity constant of human pan-IgG specific monoclonal antibodies determined by ELISA

MAbs	[Ag] (*ng/ml*)	OD-50*	[Ab] at OD-50 (*ng/ml*)	Affinity constant (M^{-1})	Average affinity constant (M^{-1})
3F2D8					
	5000	0.69	700	0.68×10^{8}	
	2500	0.64	900	0.8×10^{8}	0.74×10^{8}
	1250	0.54	920	0.75×10^{8}	
5F19G11					
	5000	1.1	6400	0.99×10^{7}	
	2500	1	7000	0.94×10^{7}	0.96×10^{7}
	1250	0.9	7500	0.95×10^{7}	

*OD-50 represents the half maximum optical density obtained for a given concentration of h-IgG ([Ag]) and the corresponding MAb ([Ab]). The affinity constant for each selected concentration of Ag and Ab was determined using the formula described in the Materials and Methods

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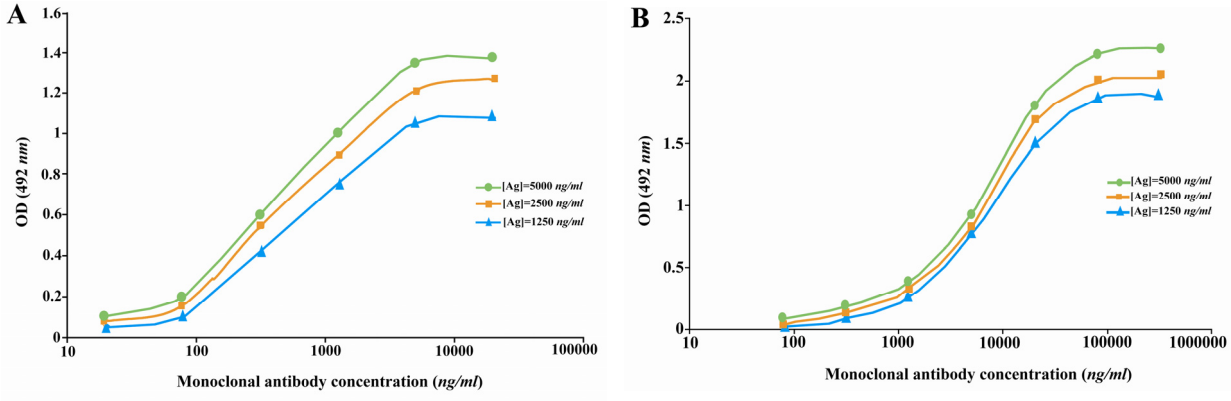

Figure 4. Representative binding curves employed for extrapolation of affinity constant of 3F2D8 (A) and 5F19G11 (B) monoclonal antibodies

displayed different profiles of cross-reactivity with a variety of animal sera. The most abundant cross-reactivity was observed with monkey Igs, while no cross-reactivity was detected with rabbit, guinea pig, sheep, goat, cat and hen sera. The animals' sera used in this study were prepared from healthy normal adult animals which naturally have normal antibody levels. Moreover, some of these sera such as sheep, goat and rabbit sera were checked with the corresponding HRP-conjugated anti-Ig antibodies as positive controls which showed strong positive signal (data not presented). Thus, lack of reactivity of the MAbs to these serum samples could not be attributed to the absence of Ig in the sera.

Cross-reactivity of anti human IgG MAbs with IgG of animal species has already been reported by other investigators [22,24,25]. Our MAbs displayed a restricted pattern of cross-reactivity. In addition to cross-reactivity with monkey serum, one of the MAbs (3F2D8) was cross-reacted with dog serum and another one (1F4C4) cross-reacted with horse serum. This pattern of cross-reactivity reflects to some extent the degree of amino acid sequence homology observed between human and other animals IgG molecules. Blasting of the human IgG protein sequence with those of the other animals reveals approximately 90% homology with monkey, 50-70% homology with other mammals and 30% with avian immunoglobulin (http://blast.ncbi.nlm.nih.gov/blast.cgi). Similar pan-IgG specific MAbs cross-reacting with horse and pig IgG, have been reported by other investigators [26]. Cross-reactivity with pig serum was not tested in our study. In another study, 13 pan-IgG Fc specific MAbs were evaluated, all of which cross-reacted with other animal sera and showed a wider pattern of cross-reactivity [24].

The affinity constant (K_{aff}) of two of our MAbs (3F2D8 and 5F19G11) was found to be $0.74\times10^{8}\ M^{-1}$ and $0.96\times10^{7}\ M^{-1}$, respectively. A similar affinity constant has already been reported for some human IgG specific MAbs using other methodologies such as agglutination inhibition [26] or fluorescent sequential saturation assay [28]. We determined the affinity of our MAbs by ELISA.

Conclusion

Pan-IgG specific MAbs are useful tools for quantitative measurement of human total or antigen specific IgG in serum in different diseases. IgG specific MAbs with relatively high affinity like 3F2D8 MAb, could enhance the sensitivity and specificity of ELISA assay for quantification of IgG. Currently, enzyme conjugated anti-IgG MAbs are used for measurement of specific IgG in a variety of ELISA kits designed for diagnosis of various infectious diseases, such as Toxoplasma [29]. Employment of a combination of pan-IgG specific MAbs with different specificities may increase the sensitivity of these assays as they complement each other via reactivity to different epitopes located to the same IgG, as

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described before [30]. These MAbs could also be used for epitope mapping of the Fc region of the IgG molecule.

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