Production and Characterization of Murine Monoclonal Antibodies Recognizing Conformational and Linear Epitopes Localized on Human IgA2 Molecules

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ABSTRACT

Background: There are two subclasses of human IgA (IgA1 and IgA2) that differ in antigenic properties and in chemical composition. The constant domains of α_1 and α_2 heavy chains have >95% sequence homology though major structural differences exist in the hinge region. Quantitation of IgA subclass levels depends on the availability of monoclonal antibodies (MAbs) specific for conserved conformational or linear epitopes restricted to each subclass. **Objective:** To produce, select and characterize monoclonal antibodies specific for human IgA2. **Methods:** Splenocytes from BALB/C mice immunized with a human IgA2 myeloma protein were fused with SP2/0 myeloma cells. Fused cells were grown in hypoxanthine, aminopterine and thymidine (HAT) selective medium and cloned by limiting dilution assay. Antibody (Ab) 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 proteins and some animal sera by ELISA and immunoblotting. The affinity constant (K_{aff}) was also determined by ELISA. **Results:** Four murine hybridoma clones designated 2F20G5, 2F20B5, 3F20E3 and 6F20H11 were obtained that secreted MAbs specific for the human IgA2. 2F20G5 and 6F20H11 MAbs react with linear epitope(s) while 2F20B5 and 3F20E3 react with conformational epitope(s) located to human IgA2 subclass. 2F20G5 MAb displays a weak cross-reactivity with monkey and rabbit sera and a strong cross-reactivity with cat and dog sera while the other three MAbs showed no cross-reactivity with the animal sera tested. **Conclusion:** These MAbs, especially 6F20H11 with high affinity constant (6.03×10^9 M^{-1}) are useful tools for quantitation of human IgA2 subclass levels in various diseases. Cross-reactivity of 2F20G5 MAb with some animal sera suggests phylogenic conservation of the epitope recognized by this MAb.

Keywords: Monoclonal Antibody, Human IgA2, Conformational Epitope, Linear Epitope, Immunoblotting, ELISA

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INTRODUCTION

IgA subclasses are distributed differently in body fluids and in cells from various tissues (1-5). Furthermore different profiles of specific IgA subclasses have been reported in a variety of diseases (6-8). There are two subclasses of human IgA (IgA1 and IgA2) that differ in antigenic properties and in chemical composition (9). Similar subclasses have been identified in certain other primates (gorilla, chimpanzee and gibbon). Most other mammals and other vertebrates in which IgA has been found have no currently known subclasses of IgA, with notable exception of the lagomorpha (rabbits and other allies) which have 13 subclasses (10). There is >95% sequence homologies between the constant domains of α1 and α2 heavy chains, but major structural differences exist in the hinge region. IgA1 molecules have an extended hinge region relative to IgA2 in which an octapeptide sequence is duplicated (11). There is a 13 amino acid deletion in the IgA2 hinge region. IgA2 shortened hinge region lacks proteolytically sensitive sites which makes it particularly resistant to cleavage by enzymes produced by a variety of bacteria that otherwise readily cleave IgA1 into its Fab and Fc fragments (12). Comparison of the amino-acid sequence of the α chains shows that seven substitutions distinguish the Cα2 domains of IgA1 and IgA2 molecules (13). IgA2 is found in at least two allotypic forms, IgA2m (1) and IgA2m (2). Solution structure of IgA2m (1) has been shown to be more compact than IgA1 (14). IgA2 constitutes 10-20% of serum IgA and 40-60% of IgA in secretions (15). Determination of IgA subclass levels in biological samples depends on the availability of monoclonal antibodies (MAbs) specific for each subclass which enhances the specificity and sensitivity of quantitative immunoassays. These subclass specific MAbs also offer an opportunity to investigate the correlation of structural variation with antigenicity. In this study we have produced and characterized four mouse monoclonal antibodies (MAbs) specific for human IgA2 with different properties.

MATERIALS AND METHODS

Collection and Purification of Human IgA Subclasses

A panel of 10 different purified human IgA myeloma proteins of known IgA subclasses and light chain types were employed in this study. This panel included seven IgA1 (MM26, MM38, MM41, MM89, MM142, MM169 and Evans) and three IgA2 subclasses, (MM161, MM81 and Cross). These myeloma proteins, obtained from patients with multiple myeloma, were purified by diethyl aminoethyl (DEAE) cellulose (Whatmann, UK) chromatography. The heavy chain and light chain isotypes and subclasses of myelomas were identified using specific mouse monoclonal antibodies including: AF6 (IgM), 8a4 (IgG), 2D7 (IgA), JA11 (IgD), C4 (λ), 6el (κ), M4D8 (IgA1) and 2E2 (IgA2), kindly provided by Professor R. Jefferis and Dr. M. Goodal (Dept. of Immunology, University of Birmingham, UK). Heavy chain and light chain of MM161 (IgA2) were separated from each other according to Lefkovits method (16) and then purified by electroelution (17). The purity of the samples was assessed by SDS-PAGE (18).

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 (8-12 weeks of age) were immunized with four intraperitoneal injections of an IgA2 myeloma protein (MM161) emulsified in Freund´s complete adjuvant (Sigma, U. S. A) (first injection) or incomplete adjuvant (Sigma) (successive 3 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 Inst. of Iran, Tehran), using polyethyleneglycol (PEG 1500) (Sigma). Hybridomas were grown in DMEM culture medium (Sigma) containing 20% fetal calf serum (FCS)(Seromed, Germany), penicillin 100 IU/ml) and streptomycin (100 μg/ml) and supplemented with hypoxantine (1×10^{-4} M), aminopterin (4×10^{-7} M) and thymidine (1.6×10^{-5} M) (HAT) (Sigma). Ten to fourteen 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 process according to the conventional methods (19). Clones secreting antibody of desired reactivity were expanded in 25 and 75 cm^2 flasks (Nunc, Denmark), harvested and cryopreserved in 40 % fetal calf serum (FCS) and 10% dimethylsulfoxide (DMSO) (Sigma).

Determination of Specificity of MAbs by Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

Microtiter polystyrene plates (Maxisorp , Nunc , Denmark) were coated with 1-10 μg/ml of purified myeloma IgA subclasses in PBS (0.15 M , PH 7.2). Then 0.05 ml of culture supernatant was added. Appropriate dilution of HRP-conjugated sheep anti mouse Ig (prepared in our lab) was then added and the reaction revealed with o-phenylenediamine dihydrochloride (OPD) (Sigma) 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.

Detection of MAbs Reactivity with Animal Sera by ELISA

Microtiter polystyrene plates (Maxisorp, Nunc, Denmark) were coated with 1/15000 dilution of animal sera in PBS (0.15 M, PH 7.2). The ELISA assay was continued as described above.

Isotype Determination of MAbs by Capture ELISA

Goat antimouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM antisera (ISO-2 kit, Sigma) at 1/1000 dilution, were adsorbed on to the wells of a microtitre ELISA plate (Nunc). 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 (20). Briefly, ELISA plates (Nunc) precoated with four different concentrations of human IgA2 ([Ag], [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 (21):

Kaff = (n-1)/2(n ([Ab′] t − ([Ab] t)

Where n= [Ag]/ [Ag′]

Immunoblotting for Analysis of Specificity of MAbs

Specificity of MAbs was assessed by Immunoblotting technique as described elsewhere [22]. Briefly, affinity purified myeloma IgA subclasses 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 containing MAbs for 1.5h at 37°C, followed by HRP-conjugated sheep anti-mouse Ig. The bands were finally visualized with diaminobenzidine tetrahydrochloride (DAB) (Sigma) substrate.

RESULTS

Screening and Selection of Specific Hybridomas

Culture supernatants from growing hybridomas were screened by ELISA using two purified IgA1 and IgA2 myelomas including the immunogen (MM161, IgA2). Four hybridomas secreting MAbs specific for human IgA2 were identified (Table 1).

Table 1. Reactivity of representative hybridomas with selected IgA subclasses

Screening Antigens	OD (492 nm)	
Hybridomas	**IgA1 (MM142)**	**IgA2(MM161)**
6F20H11	0.04	2
2F20G5	0.03	0.7
3F20E3	0.06	2
2F20B5	0.02	0.8
Anti IgA1 (M4D8)	0.9	0.2
Anti IgA2 (2E2)	0.25	1

Characterization of Specific MAbs

Following cloning and subcloning, all four MAbs were found to express IgG1 subclass (Table 2). Specificity of the MAbs was determined using a panel of purified myeloma proteins including IgA1 (n=7) and IgA2 (n=3) subclasses. Our results demonstrated that all the MAbs were specific for isotypic epitopes located on IgA2 subclass (Figure 1). Among these MAbs, 2F20G5 reacted only with heavy but not light chains and the others showed no reactivity with either light or heavy chains of the immunizing protein (MM161), as shown by ELISA (Figure 2).

Table 2. Determination of the isotype of MAbs by ELISA

MAbs / Isotype	2F20G5	2F20B5	3F20E3	6F20H11	Control⁻
IgG1	0.76	0.6	0.86	0.73	0.16
IgG2a	0.2	0.2	0.2	0.2	0.19
IgG2b	0.18	0.16	0.17	0.2	0.17
IgG3	0.15	0.15	0.16	0.18	0.15
IgA	0.2	0.2	0.2	0.2	0.12
IgM	0.2	0.2	0.2	0.17	0.18

Control: Culture supernatant from SP2/0 myeloma cells

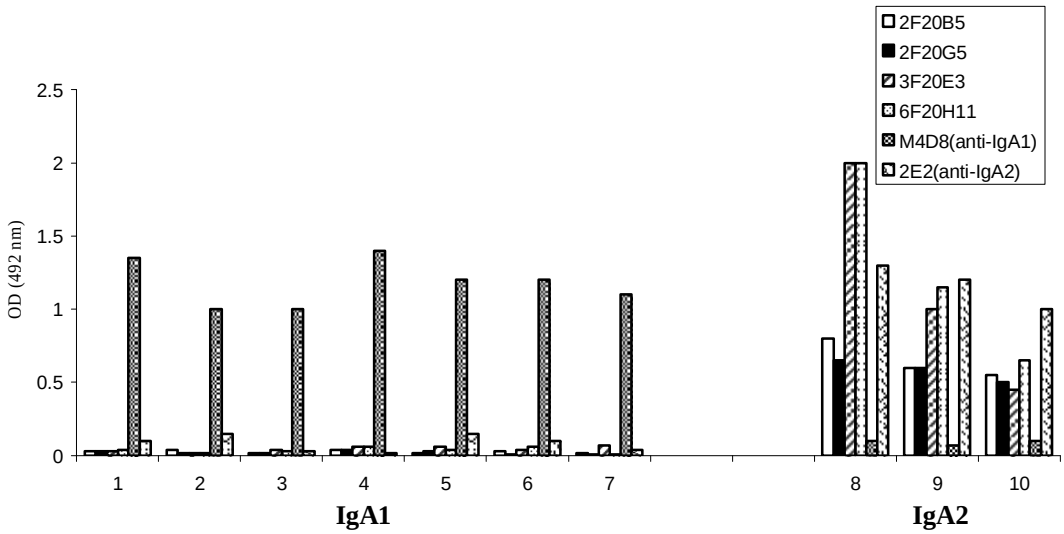

Figure1. Reactivity of mAbs with IgA subclasses
Lanes 1 to 10 represent: MM26, MM38, MM41, MM89, MM142, MM169, Evans, MM161, MM81 and Cross, respectively.

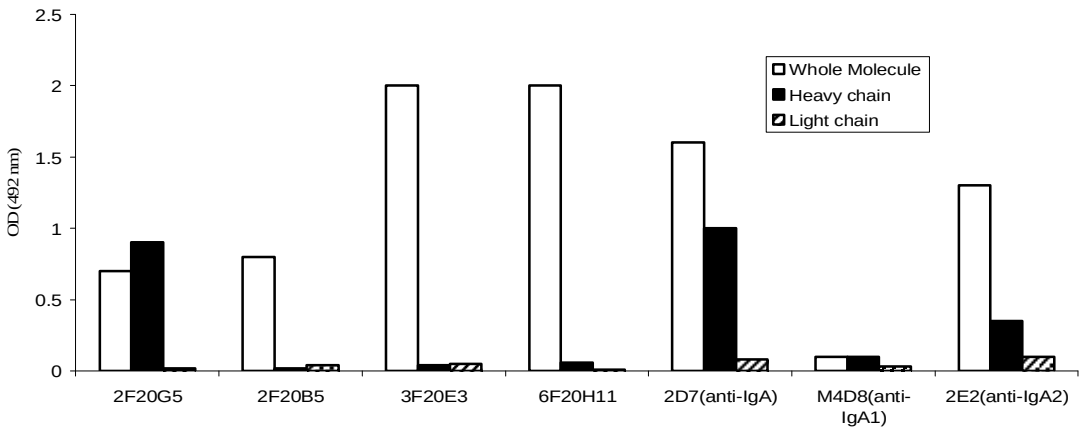

Figure2. Reactivity of MAbs with IgA2 (MM161) and its chains.

Immunoblotting studies demonstrated that 2F20G5 and 6F20H11 MAbs recognize sequential epitopes (Figure 3A) while 2F20B5 and 3F20E3 recognize conformational epitopes (Figure 3B) located on human IgA2 heavy chain.

Cross-reactivity studies employing whole sera from a range of animal species indicate that only one of our MAbs (2F20G5) shows cross-reactivity with some animal sera. 2F20G5 has a strong cross-reactivity with cat and dog sera, a weak cross-reactivity with monkey and rabbit sera and no cross-reactivity with guinea pig, sheep, horse, goat and hen sera (Table 3).

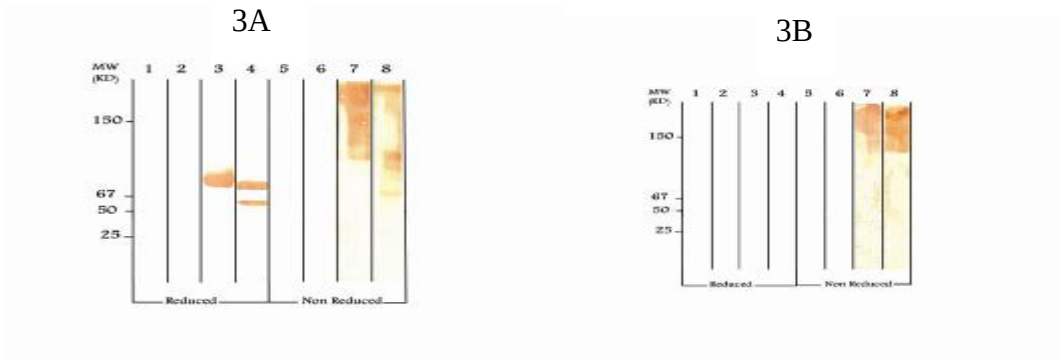

Figure 3A. Immunoblot analysis of 6F20H11 MAb with IgA subclasses.
Lanes 1 to 4 represent reduced forms of Evans (IgA1) ,MM26 (IgA1), MM161 (IgA2) and MM81 (IgA2,) respectively. Lanes 5 to 8 represent non-reduced forms of Evans (IgA1) ,MM26 (IgA1), MM161 (IgA2) and MM81 (IgA2), respectively.
Figure 3B. Immunoblot analysis of 2F20B5 MAb with IgA subclasses. See footnotes of Figure 3A.

Table 3: Cross-reactivity of mAbs with animal sera

Animal sera	%Cross-reactivity*			
	2F20G5	2F20B5	3F20E3	6F20H11
Monkey	9	<0.1	<0.1	<0.1
Rabbit	23	<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	96	<0.1	<0.1	<0.1
Dog	95	<0.1	<0.1	<0.1
Horse	<0.1	<0.1	<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.

Determination of the Affinity of 6F20H11 MAb

Among the four IgA2 specific MAbs obtained in this study, the ascitic fluid was available only for 6F20H11 MAb. This MAb was purified by affinity chromatography using streptococcal protein G column and the affinity constant (K_{aff}) was determined by ELISA. Triple serial concentrations of the antigen (IgA2) and MAb were selected to construct the corresponding curves and extrapolate the K_{aff} values using the formula given in Materials and Methods. Representative curves obtained for 2F20G5 MAb are illustrated in Figure 4 and the calculated average K_{aff} value are presented in Table 4.

Table 4. Affinity Constant of 6F20H11 mAb determined by ELISA

[Ag] (ng/ml)	OD-50*	[Ab] at OD-50 (ng/ml)	Kaff(M^{-1})	Average Kaff(M^{-1})
2500	1.11	50	1.5×10^9	
1250	1.025	52	1.07×10^9	1.22×10^9
625	0.96	61	1.15×10^9	
312.5	0.825	63	1.15×10^9	

*****OD-50 represents the half maximum optical density obtained for a given concentration of h-IgA2 ([Ag]) and the corresponding MAb ([Ab]). The affinity constant (K_{aff}) for each selected concentration of Ag and Ab was determined using the formula described in the Materials and Methods.

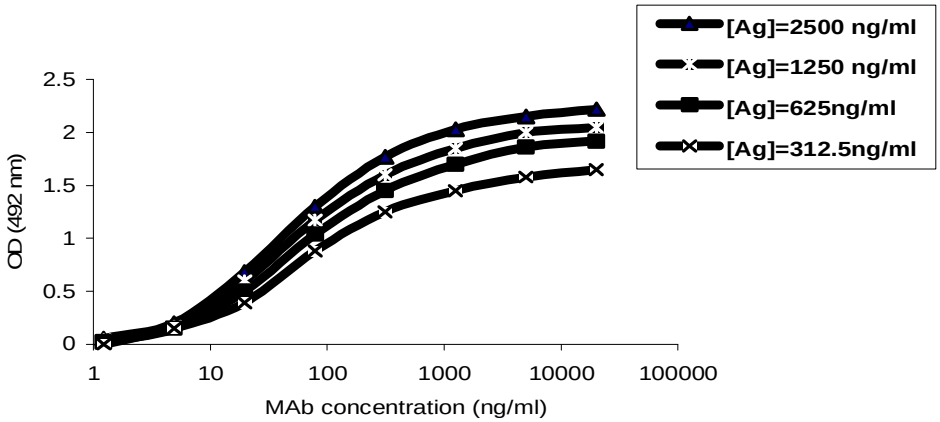

Figure 4. Representative binding curves employed for extrapolation of affinity constant of 6F20H11 MAb.

DISCUSSION

In the present study, production and characterization of four mouse MAbs specific for human IgA2, has been described. A number of investigators have produced murine MAbs specific for human IgA2 subclass (9, 23-27). In an IUIS/WHO collaborative study, 51 MAbs with putative specificity for different human IgA epitopes, were evaluated for immunoreactivity and specificity by nine laboratories employing imunodiffusion, agglutination, immunohistochemistry, immunoblotting and ELISA immunoassays (12). Of the 51 original MAbs, only three (HP6109, 2E2 and 512-H5.1) reacted with both allotypic forms of IgA2. Of these, HP6109 and 512-H5.1 possessed a higher affinity for IgA2 while 2E2 MAb appeared to be less potent as a result of its lower affinity (12). In the case of HP6109 MAb contradictive results have also been reported indicating lower affinity levels (26).

Localization of the epitopes recognized by these MAbs revealed different specificities (9, 12, 26, 28). Accordingly, α chain domain epitope locations for HP6109 and 512-H5.1 MAbs, are IgA2-(CH2)2 and IgA2-CH2 respectively. These MAbs all belonged to IgG1 isotype (12) similar to our MAbs. Studies conducted by Faris et al. (23) regarding cross-reactivity profile of 2E2 MAb indicated that it cross-reacts with serum immunoglobulin from monkey, rabbit and cat (like our MAb, 2F20G5), guinea pig (unlike our MAb, 2F20G5), BALB/C mouse, NZB mouse, rat, bovine, donkey and gorilla. The reactivity of 2E2 MAb (produced in BALB/C mice) with BALB/C mice serum is a little bit surprising because animals are tolerant to their own antigens (self antigens) (29). The strong cross-reactivity of our MAb, 2F20G5 with cat serum is in agreement with other results reporting extensive conservation between human and cats` genomes (30, 31) including $C\gamma_1$ sequence (31). Conservation also exists between human and cats` in $C\alpha_2$ gene sequence as indicated by our results. Strong cross-reactivity of 2F20G5 with dog serum, may suggest reactivity with dog IgA. Although the single IgA subclass so far identified in dog has an alpha-chain hinge region with a predicted amino-acid sequence similar to that of human $alpha_1$ chain, four sequence variants were identified with a shortened hinge region in two of

the variants (32). Thus 2F20G5 MAb, with IgA2 specificity may recognize an IgA allotypic marker in dog.

Reactivity of our MAb, 2F20G5 with denatured α_2 heavy chain (reduced IgA2 under 2-ME treatment in immunoblotting and also electroeluted α_2 heavy chain in ELISA), clearly shows that a stable linear epitope is recognized by this MAb. We used 2E2 as a control and it showed weak reactivity with electroeluted α_2 heavy chain (Figure 2), perhaps because of its low affinity. Amino acid sequence analysis performed by other investigators suggested that most of the IgA subclass specific MAbs with specificity for Fc region, react with conformational CH2 domain epitopes (26). Due to unavailability of Fc and Fab fragments as well as domain deleted molecules of IgA2, we have not been able to assign domain specificity of our MAbs. Immunoblotting results, however demonstrated that two of the MAbs (2F20G5 and 6F20H11) reacts with linear epitopes, whereas two other MAbs recognize conformational epitopes lost upon heavy chain reduction (Figures 3A and 3B**).** Lack of reactivity of 6F20H11 MAb with electroeluted heavy chains of the immunizing protein by ELISA as opposed to the immunoblotting results may partly be explained by the prolonged process of purification and isolation of the electroeluted heavy chains which could have resulted in progressive proteolytic degradation (33) or denaturation leading to loss of the target epitopes.

These MAbs in conjunction with the limited number of previously generated IgA2-specific MAbs may prove to be valuable tools for epitope mapping of human IgA2 and quantitative measurement of this subclass in biological samples.

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