Measurement of Affinity Constant of Anti-human IgG Monoclonal Antibodies by an ELISA-based Method

Fatemeh Hajighasemi[1,2], Ali Akbar Saboor-Yaraghi[1], Fazel Shokri[1,3*]

[1]Department of Immunology, School of Public Health, Tehran University of Medical Sciences, [2]Department of Immunology, School of Medicine, Shahed University, [3]Monoclonal Antibody Research Center, Avicenna Research Center, Tehran, Iran.

ABSTRACT

Background: The affinity of an antibody to its antigen is a crucial parameter in its biological activity and performance of an immunoassay such as ELISA. Affinity of most IgG specific MAbs are often determined by methods which require labeling of either antigen or antibody, and are sometimes difficult to control, do not always lead to the expected signal and often result in immunological modification of the molecules. Moreover, direct solid phase binding assays pose some problems such as diffusion effects and difficulties in reaching equilibrium due to heterogeneous binding and co-operativity. **Objective:** To employ a rapid and simple ELISA-based method for measuring affinity constants of two pan-h-IgG specific MAbs (3F2D8 and 5F19G11) established in our laboratory. **Methods:** The method is based on the effect of antibody affinity on the sigmoidal dose response curve. In this method, the binding of anti-human IgG (anti-h-IgG) MAbs with their corresponding antigen was measured using serial concentrations of both antigen and antibody. The amount of antibody bound to the antigen on the plate is represented as a sigmoidal curve of OD versus the logarithm of antibody concentration added to each well. **Results:** Based on the data obtained from this study, the affinity constants of 3F2D8 and 5F19G11 MAbs were 0.74×10^{8} Mol $^{-1}$ and 0.96×10^{7} Mol $^{-1}$, respectively. **Conclusion:** 3F2D8 MAb with reasonably high affinity is suggested as a candidate for quantitative measurement of IgG by ELISA, whereas 5F19G11 MAb could be considered as a suitable tool for immunoaffinity chromatography.

Key words: Affinity Constant, ELISA, Human IgG, Monoclonal Antibody

*Corresponding author: Dr. Fazel Shokri, Department of Immunology, School of Public Health, Tehran University of Medical Sciences, P.O. Box: 6446-14155, Tehran, Iran. Tel: (021) 6462268, Fax: (021) 6462267, e-mail: fshokri@sina.tums.ac.ir

INTRODUCTION

The concentration of total or antigen specific IgG changes in many pathological conditions (1-3). Preparation of specific polyclonal reagents for detection of immunoglobulin isotypes is tedious, requiring multiple adsorption processes often incomplete resulting in residual cross reactivity (4). Monoclonal antibodies (MAbs) specific for human IgG are useful tools for measurement of total and antigen specific IgG. MAbs used in diagnostic methods, should be characterized in terms of several physiochemical parameters (5). The affinity of an antibody to its antigen is a crucial parameter in the biological activity of antibody (6,7) and performance of an immunoassay such as ELISA (8,9). High affinity anti-IgG MAbs, enhance the specificity and sensitivity of ELISA assay for quantitative detection of IgG. Affinity of most IgG specific MAbs has been determined by radioimmunoassay (5,10) or sequential-saturation immunofluorescent method (11). These methods require labeling of either antigen or antibody, which is sometimes difficult to control, does not always lead to the expected signal and often results in immunological modification of the molecules (12). To circumvent these problems, homogenous liquid phase methods in which complex formation of antigen and antibody takes place in solution, have been replaced to a large extent by heterogeneous solid phase methods where one of the reactants is immobilized (8). The kinetics of solid-phase antigen-antibody reactions differs significantly from classical liquid phase reactions (13). In solution the interaction between a hapten and an antibody is reversible and not limited by any diffusion or multivalent binding effects. It therefore follows the basic thermodynamic principles of a non-covalent bimolecular interaction. Intrinsic affinity (true affinity) which refers to the interaction between a single antigenic determinant and a single antibody-combining site can be analyzed in conventional mass-action terms in equilibrium binding experiments (14). Multivalence of either antigen and/or antibody makes the determination of intrinsic affinity impossible and hence, the term functional affinity (avidity) is applied for multivalent antibody and antigen (15). In the present study, functional affinity refers to the interaction of an immobilized antigen with its corresponding antibody without the multivalent effect of either reactant.

There are some problems in direct solid phase binding assays, which complicate the determination of affinity (16,17). Diffusion plays an important role in heterogeneous binding by regulating the association and dissociation rate, thus reaching the equilibrium. Moreover, modification of the antigen/antibody interaction due to immobilization of the antigen (18), surface effects such as antigen-density dependent steric hindrance, bivalent antigen binding and lateral co-operative interactions between antibodies can influence the estimation of the affinity constant (16,19,20).

In this study a solid-phase ELISA-based method was employed to measure functional affinity of two anti human IgG MAbs established in our laboratory. The method adapted from Beatty et al. (21) is based on the effect of antibody affinity on sigmoidal dose response curve and measures the affinity constant of MAbs employing the Law of Mass Action. This method of affinity determination is rapid, reliable and easy to perform.

MATERIALS AND METHODS

Antigen. The antigen, a human IgG1 Kappa myeloma protein (MM1) was obtained from a patient with multiple myeloma and purified by diethyl aminoethyl (DEAE) cellulose (Whatmann, UK) chromatography. The heavy and light chain isotypes and subclasses of the myeloma protein were determined using specific murine MAbs including: AF6 (IgM), 8A4(IgG), 2D7(IgA), JA11 (IgD), C4 (λ), 6E1 (κ), JL512 (IgG1), GOM2 (IgG2), ZG4 (IgG3) and RJ4 (IgG4), kindly provided by Professor R. Jefferis and Dr. M. Goodal (Deptartment of Immunology, University of Birmingham, UK).

Monoclonal antibodies. Two mouse anti h-IgG MAbs designated 3F2D8 and 5F19G11 were selected from a large panel of mouse anti-h-IgG MAbs, developed in our lab. We produced and characterized the MAbs as described in detail previously (22). Both of the MAbs belonged to IgG1 subclass and were specific for linear epitopes located on Fc fragment of all IgG subclasses. The MAbs were purified by affinity chromatography using Staphylococcal protein A (SPA) or Streptococcal protein G (SPG) Sepharose 4B (Pharmacia, Uppsala, Sweden) from ascetic fluids of Balb/c mice inoculated by the corresponding hybridoma cells. The purity of antibody preparations was monitored by SDS-PAGE according to Laemmli (23).

Affinity constant determination

ELISA procedure. The method described here is adapted from Beatty et al. (21), with some modifications. This ELISA procedure was set up by performing a series of optimization experiments. All reactions were performed in sealed (to prevent evaporation) microtitre polystyrene plates (Maxisorp, Nunc, Denmark) with all reaction volumes of 50 µl. Plate washing was performed three times after each incubation with PBS (0.15 M, PH = 7.2) containing 0.05% Tween 20 (Sigma) (PBS/T) for 1.5 hr at 37ºC. The plates were freshly coated with serial dilutions of purified myeloma IgG, in PBS (0.15M, PH = 7.2). The IgG concentrations employed in the various coating solutions were 20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.78, 0.39 and 0.19 µg/ml. Then serial dilutions of MAbs (80-0.02 µg/ml) in PBS/T were added into each coated well (dilution factor, 1:4) and incubated for 1.5 hr at 37º C. After washing, appropriate dilution of a home-made horseradish peroxidase (HRP)-conjugated sheep anti-mouse Ig was added and the plates were incubated for 1.5 h at 37º C. Following the final washing step, the reaction was revealed with O-Phenylendiamine Dihydrochloride (OPD) (Sigma) substrate. Finally, the reaction was stopped with 20% H2SO4 and the optical density (OD) was measured by a multiscan ELISA reader (Organon Teknika, Belgium) at 492 nm.

Calculation of affinity constant. The amount of antibody bound to the antigen on the plate is represented as a sigmoidal curve of OD versus the logarithm of antibody concentration added to each well. The antibody concentration resulting in 50% of the maximum absorbance value at a particular antigen coating concentration was selected for the affinity calculation. According to Beatty et al. (19), equation (1) is an estimate of the affinity constant of the antigen-antibody interaction that is based solely upon the antibody concentration at OD_{50} of ($[Ab']_t$ and ($[Ab]_t$) for plates

coated with two amounts of antigen, one ([Ag′]) being half the other ([Ag]). Equation (2) is a general formula derived from equation (1) when different concentrations of [Ag] ([Ag] = n [Ag′]) are used, one being half the other (e.g. Ag = 2Ag′ = 4Ag′′= 8 Ag′′′).

$K_{aff} = 1/2(2[Ab']_t - [Ab]_t)$ (1)

$K_{aff} = (n-1)/2(n[Ab']_t - [Ab]_t)$ (2)

Where n = [Ag] / [Ag′]

The final affinity constant measured in this study is the mean of three calculations as illustrated in table 1. Each MAb concentration at OD_{50} of a particular sigmoid curve, selected for calculation according to the above criteria, was used twice (and not three times as in the Beatty method) to calculate the final functional affinity constant.

RESULTS

Optimizing the antigen coating conditions. The antigen coating conditions are critical in the Beatty method (21) for determining the functional affinity constants. Doubling the antigen concentration should make an increase in solid-phase coating by a factor of two, which is essential for the validity of mathematical equation used for calculations.

We used a solid-phase non-competitive coating procedure and selected three h-IgG coating concentrations [5, 2.5 and 1.25 μg/ml (ratio of 1:2:4)] producing appropriate final OD values in the ELISA from the linear portion of the serial h-IgG dilution curves (Fig.1).

Table 1. Affinity constants of h-IgG specific MAbs determined by ELISA.

MAbs	*[Ag] (ng/ml)*	*OD-50**	*[Ab] at OD-50 (ng/ml)*	*Kaff(M^{-1})*	*Average Kaff(M^{-1})*
3F2D8	5000	0.69	700	0.68×10^8	**0.74×10^8**
	2500	0.64	900	0.8×10^8	
	1250	0.54	920	0.75×10^8	
5F19G11	5000	1.1	6400	0.99×10^7	**0.96×10^7**
	2500	1	7000	0.94×10^7	
	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 (K_{aff}) for each selected concentration of Ag an Ab was determined using the formula described in the Materials and Methods.

Experimental dose response curves for anti h-IgG MAbs. This ELISA method utilizes the influence of antibody affinity on the sigmoidal solid-phase ELISA curves for determining the functional affinity constant (21). For each coating solution, serial dilutions of each of the two monoclonal antibodies specific for human IgG

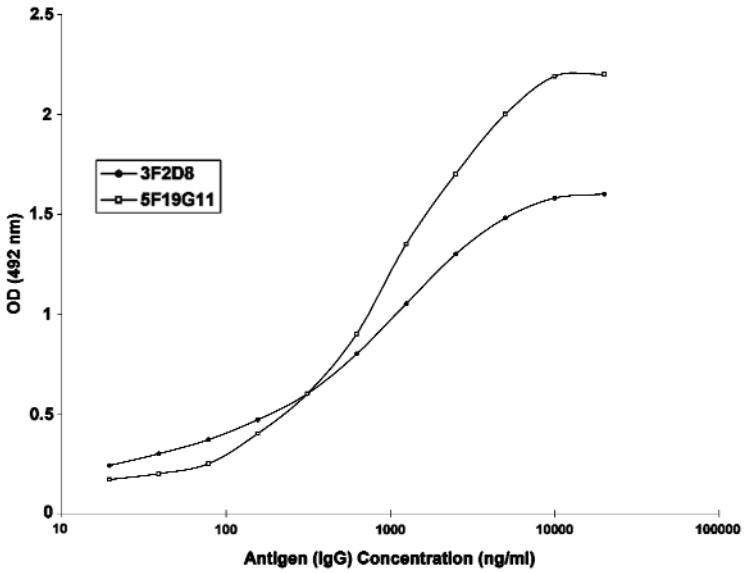

Figure 1. Representative sigmoidal curves of IgG-specific MAbs using serial dilutions of coating antigen (hIgG). MAbs 3F2D8 and 5F19G11 (80 µg/ml) were used in excess to achieve saturation

were applied to different rows on the microtitre plate. Each row was tested in duplicate and the antibody concentration at OD_{50} of the sigmoid curve was measured for each h-IgG concentration. To obtain a complete sigmoid curve of anti-h-IgG MAbs, serial dilutions of the MAbs ranged from 0.02 to 80 µg/ml were chosen. Experimental serial dilution curves of our MAbs using three different h-IgG coating concentrations are shown in figures 2 and 3.

Calculation of the functional affinity constant. Affinity constant of the two anti-h-IgG mouse MAbs was calculated by the general equation (2) given in the Materials and Methods. Based upon the curves in figures 2 and 3, the mean K_{aff} for 3F2D8 and 5F19G11 MAbs were found to be 0.74×10^8 Mol $^{-1}$ and 0.96×10^7 Mol $^{-1}$, respectively. The results are summarized in table 1.

DISCUSSION

Antibody affinity has important implications in the performance of an immunoassay such as ELISA (8,9). In this study we employed a rapid and simple ELISA-based method adapted from Beatty et al. (21) utilizing the effect of antibody affinity upon the sigmoidal dose response curve to measure affinity constants of two Pan-h-IgG MAbs (3F2D8 and 5F19G11) established in our lab. This method is based upon the Law of Mass Action which determines the binding strength between a MAb and its immobilized antigen by comparing the OD_{50} of two sigmoid curves of antibody serial dilutions on plates coated with two different antigen concentrations. Direct solid phase binding assays pose some problems such as:

1) Kinetic changes due to diffusion effects which decreases the probability of

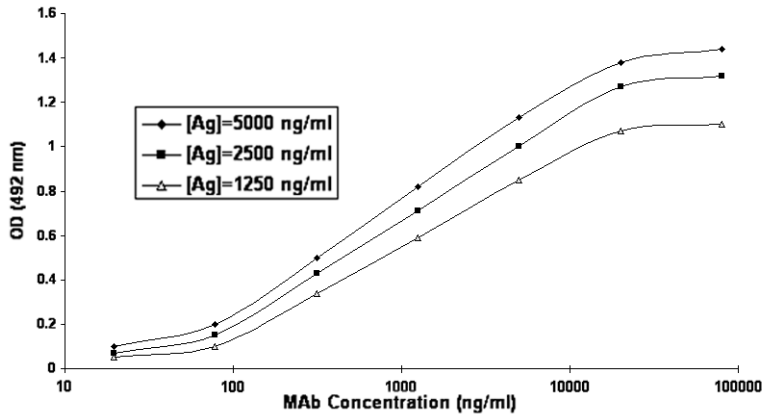

Figure 2. Experimental dose-response curve for anti hIgG monoclonal antibody 3F2D8 at three different hIgG concentrations

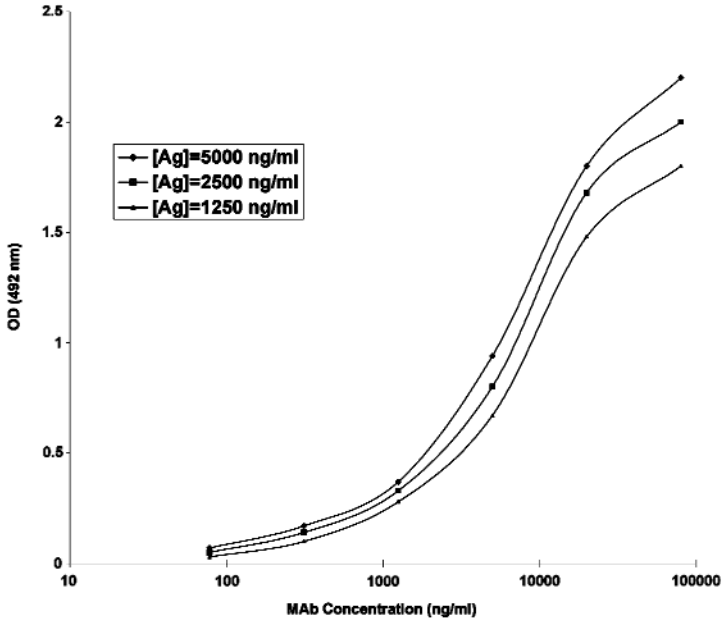

Figure 3. Experimental dose-response curve for anti hIgG monoclonal antibody 5F19G11 at three different h-IgG concentrations

reaching an equilibrium.
2) Occurrence of co-operativity (lateral Fc interaction and binding site heterogeneity).
3) Steric hindrance conditions such as epitope density and especially the frequency of bivalent binding.
Under such conditions, the interaction between antigen and antibody does not obey the Law of Mass Action and the use of Beatty formula becomes controversial.
Several studies have reported that experimental conditions such as epitope density and especially the frequency of bivalent binding could be the cause of all the above-mentioned solid-phase problems (9,14,16,17,24,25). It has recently been reported

that the bivalent binding of anti-h-IgG MAbs with immobilized h-IgG does not occur (26) and the absence of bivalent binding was ascribed to relatively large size of the h-IgG molecules and its sticky structure. Thus monovalent binding between h-IgG and its MAb, under equilibrium conditions, permit application of the Law of Mass Action and hence the Beatty formula. The Beatty formula has also been justified for assessment of functional affinity constant of anti human chorionic gonadotropin (HCG) MAbs by examining and confirming monovalent binding between HCG and its MAb (8).

In the present study existence of equilibrium condition was based on the assumption of monovalent binding between h-IgG and its MAb (26) as well as the presence of active free antibody and immobilized antigen molecules as could be found in figures 2 and 3. The figures show three experimental sigmoid dose-response curves for different h-IgG coating concentrations. The increase in OD values observed between the different sigmoid curves at one particular antibody concentration demonstrated the presence of free active antibody molecules. In a similar way, the presence of active immobilized antigen under equilibrium conditions was shown by the increase of the OD values on a single sigmoid curve (one particular h-IgG coating concentration) as the MAb concentration increases. These results are consistent with those of Beatty (21) and Loomans (8).

The other important problem is that immobilization of the antigen may modify the antigen/antibody interaction and so affinity by altering the conformation of the protein (18,27). Nevertheless, if the MAb is employed in solid phase methods with immobilized antigen, affinity measurement offers the possibility of determining the natural affinity constant.

In conclusion we employed a simple method for assessment of the functional affinity constant of two anti h-IgG MAbs with advantages to methods previously used:

1) Determining the natural affinity constant because MAbs are usually employed in solid phase methods such as hybridoma screening.

2) Requires no labeling of the antibody or antigen.

3) Permits simply and rapidly estimating the K_{aff} of many MAbs binding monovalently to the same antigen.

Knowing the K_{aff} of an antibody allows judicious selection of antibody for a specific purpose, such as using low affinity antibody in an affinity chromatography procedure for antigen purification (28) or high-affinity antibody in immunoassay techniques (9). Employment of low affinity antibody in an immunoaffinity column would allow purification and elution of the bound antigen with a mild elution buffer avoiding protein denaturation. The use of a high affinity MAb, on the other hand, is regarded as a decisive factor for improvement of the sensitivity of an immunoassay, such as ELISA. Thus the MAb 5F19G11 which displays a lower affinity constant (0.96×10^7 Mol $^{-1}$) compared to 3F2D8, may prove to be suitable for the former application (that is affinity chromatography). The MAb 3F2D8, however, with almost 10 times higher affinity constant, seems to be more suitable for development of immunoenzymatic detection and quantification assays.

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