

Determination of kinetic parameters of epitope-paratope interaction based on solid phase binding: An inexpensive alternate to biospecific interaction analysis

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MS received 5 June 1996

Abstract. A method has been developed for biospecific interaction analysis between antigen and antibody using solid phase binding approach. Real time kinetics between monoclonal antibody and human chorionic gonadotropin have been studied. Kinetic constants of the bimolecular reaction are determined. Affinity constants measured by several independent methods have been found to be relatively consistent. Convenient and simple procedures to determine affinity constant, K_{on} and K_{off} of monoclonal antibody-human chorionic gonadotropin interaction using binding of [125 I]hCG to immobilized monoclonal antibody are presented. Values obtained compare well with those obtained using surface plasmon resonance technology, making this method a viable alternative.

Keywords. Antigen-antibody interaction; association rate constants; dissociation rate constants; affinity constant; biospecific interaction analysis; human chorionic gonadotropin.

1. Introduction

Widespread use of monoclonal antibodies (MAb) as probes in physiological studies and in various lines of research like epitope mapping have made it necessary to characterize the epitope-paratope interaction. A unique feature of MAb-antigen (Ag) interaction is its true bimolecular nature, where each molecule of Ag combine with a single MAb paratope, unlike in the polyclonal Ab-Ag system involving simultaneous interaction with multiple paratopes. One of the most important characterization of an equilibrium reaction like MAb-Ag system would be the study of kinetics of interaction involving determination of rate of forward and backward reactions and affinity constants. These kinetic parameters have been analysed extensively in other macromolecular interactions like enzyme substrate reaction, oligomeric assembly etc. However investigations on dynamics of reaction between MAb and antigen have been few (Raman *et al* 1992; Larvor *et al* 1994) and recently with the advent of BIAcore system (Pharmacia) several determinations have been done (Malmquist 1993a, b; Altschuh *et al* 1992; Pellequer and Van Reganmortel 1993; Gruen and Kortt 1994; Ichiyoshi *et al* 1995; George *et al* 1995). One of the most frequently determined equilibrium kinetic constant, namely affinity constant has been measured for MAb-Ag pairs using ELISA methods, but the results tends to be inconsistent (Underwood 1993). In our studies we have observed that several MAb-Ag complexes dissociate significantly during multistep assay formats. Use of solid phase radioimmunoassay (SPRIA) arrests the reaction at equilibrium, and hence eliminates the uncertainty of multistep ELISA

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methods (Venkatesh and Murthy 1996). In another study we have also shown that paratope (MAb) when immobilized can be quantitated using conventional Scatchard plot of SPRIA data, and hence the paratope density on the microtiter well can be determined with certainty (G S Murthy, unpublished results). Thus both the uncertainties associated with the ELISA approach for kinetic studies are eliminated when SPRIA method is adopted. In the present report we have investigated the kinetics of the reaction of several epitope-paratope pairs, namely hCG and its MAbs using solid phase binding (SPB) to measure kinetic parameters of the interaction.

2. Materials and methods

2.1 Materials

hCG, hLH and hFSH were gifts from NHPP program. Iodination of the hormones was done by iodogen method (Fraker *et al* 1978). Specific activity of the iodinated hormone was calculated by the extent of incorporation and was always expressed as cpm/ng. For Scatchard plot and other kinetic experiments same stock of hCG was used for both iodination as well as displacement analysis in SPRIA. All experiments were done with freshly prepared iodinated hormones which showed more than 80% precipitability with polyclonal antisera indicating minimal damage to the hormones due to iodination. Specific activity of [¹²⁵I]hCG was between 40–45,000 cpm/ng and of [¹²⁵I]hFSH 80–85,000 cpm/ng. Extent of precipitability of hCG was 95% while that of hFSH was 85%. This correction was incorporated in all calculations. MAbs VM4, VM7 and VM9 for hCG $\alpha\beta$ were produced in the laboratory and their characteristic will be reported later. MAb D-68 is a gift from Dr J Dias, Wadsworth Research Centre, USA.

2.2 SPRIA

SPRIA was carried out as described earlier (Venkatesh *et al* 1995; Murthy and Venkatesh 1996) using MAbs immobilized on plastic wells through immunochemical bridge. MAbs were coated at concentration of 1/50 to 1/500 (250 μ l/well) overnight for immobilization. Briefly 125 μ l of varying concentration of hCG (ranging from 8 ng/ml to 1000 ng/ml) and 125 μ l of [¹²⁵I]hCG were incubated in MAb coated wells for 20 h. Binding was terminated by aspirating the supernatant and the bound complex was washed once with RIA buffer and counted in a multigamma counter.

2.3 Binding of [¹²⁵I]hCG to solid phase MAbs

A set of wells coated with MAb was incubated with 50–100,000 cpm of [¹²⁵I]hCG in a total volume of 250 μ l. At different time points binding was terminated by discarding unbound [¹²⁵I]hCG and washing the wells with RIA buffer. At each point of time duplicate wells were processed. At the end of the experiment wells were counted in a gamma counter. Using the hormone binding capacity of the wells (from Scatchard plot or single point data) and the specific activity of the [¹²⁵I]hCG second order rate constant of the reaction K_{on} was calculated using the formula

$$K_{on}.t = [1/(a-b)]. [\ln \{b(a-x)/a(b-x)\}],$$

where b = capacity of the well to bind hormone, a = initial concentration of [^{125}I]hCG, x = slope of the linear plot (mol/l) and t = time of reaction.

2.4 Determination of the first order rate constant of dissociation

Binding of [^{125}I]hCG to immobilized MAb was carried out for 2–3 h at room temperature. After terminating the reaction, followed by washing with RIA buffer bound radioactivity was measured in a gamma counter. To each well was added 250 μl of RIA buffer containing 250 ng of hCG to start the dissociation, and at different points of time it was arrested by discarding the contents of the well. Residual radioactivity was measured and first order rate constant of the dissociation K_{off} was obtained by the formula

$$K_{\text{off}} t = \ln [(a-b)/(x-b)],$$

where a = cpm bound at time 0 (start of dissociation), x = cpm bound at time t (represents undissociated cpm), b = cpm still bound after 20 h (nonreversibly bound) and K_{off} = first order rate constant of dissociation.

2.5 Affinity constant measurements

Affinity constant measurements were done by different methods.

(i) Scatchard analysis of the SPRIA data by standard procedure.

(ii) *Single point binding data*: In this method [^{125}I]hCG was bound to MAb coated well overnight, and using the capacity obtained by Scatchard plot (b), K_a was measured by the following formula

$$K_a = [x/(a-x)(b-x)],$$

where a = initial concentration of [^{125}I]hCG, b = initial concentration of MAb/capacity of the well to bind the hormone, and x = concentration of [^{125}I]hCG-MAb complex at time t with all concentrations expressed as mol/l.

(iii) *K_a by single point dissociation data*: In this method [^{125}I]hCG was bound to solid phase MAb for 5 h or 20 h and the well were washed. This solid phase MAb-[^{125}I]hCG complex was allowed to re-equilibrate with 250 μl of RIA buffer overnight and the radioactivity bound to the well determined. K_a was calculated from the formula

$$K_a = [x/(c-x)(b-x)],$$

where x = [^{125}I]hCG bound to the well after re-equilibration, b = capacity of the well to bind hormone, c = [^{125}I]hCG bound at the beginning of re-equilibration ($t = 0$) with all concentrations expressed as mol/l.

(iv) K_a was also calculated from the formula

$$K_a = [K_{\text{on}}/K_{\text{off}}],$$

where K_{on} and K_{off} represent the forward and backward reaction rate constants (obtained as outlined above).

3. Results

Displacement profile of [^{125}I]hCG in MAb SPRIA is shown in figures 1 and 2 for MAbs VM 4, D-68, VM 7 and VM 9. Insets present respective Scatchard plot analysis

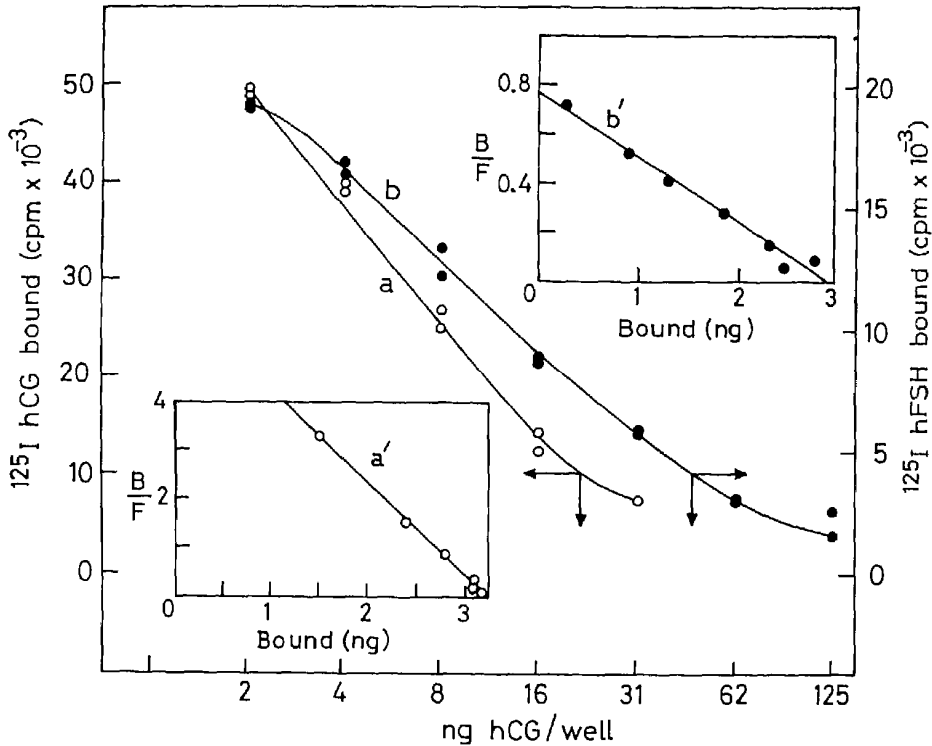


Figure 1. Displacement of ^{125}I hCG (curve a) and ^{125}I hFSH (curve b) from solid phase MAbs (VM 4 and D68) by hCG and hFSH respectively. Inset: Scatchard plots from this SPRIA (curve a' for VM 4 and curve b' for D-68). The wells were coated at a dilution of 1/500 for VM 4 and 1/20000 for D-68.

of SPRIAs. The plots are linear and affinity constants have been measured from these data. K_a obtained by different methods from these data is shown in table 1. It is seen that K_a measured by single point dissociation method is different from either Scatchard plots or single point binding method, and indicates that when specific binding is very high the discrepancy reduces (last row table 1)

Kinetics of dissociation of epitope-paratope pair in figure 3 shows the reaction (inset in figure 3) fitting into first order kinetics. In dissociation experiments extent of dissociation observed in the presence of excess of hCG (100-fold) was only 80%. Rest (20%) remained undissociated even after additional 20 h of incubation. Linear plot in the inset of figure 3 has been used to calculate the first order rate constant and is found to be 0.05/min and 0.06/min for VM 7 and VM 9 respectively. Rate of binding of ^{125}I hCG to VM 7 and VM 9 are shown in figure 4. The binding increases linearly with time. Its slope has been used to calculate K_{on} and the values obtained are 1.4×10^7 and 0.8×10^7 respectively for the two MAbs. Comparison of affinity constants obtained for these two MAbs by Scatchard plot, single point association and by the forward and backward rate constants is compared in table 2. It is seen that while the data of single point binding and Scatchard plot agree well those obtained by the forward and backward rate constants are lower to Scatchard values by 40–70%. This may indicate that the kinetics is only apparently second order, and the differences may

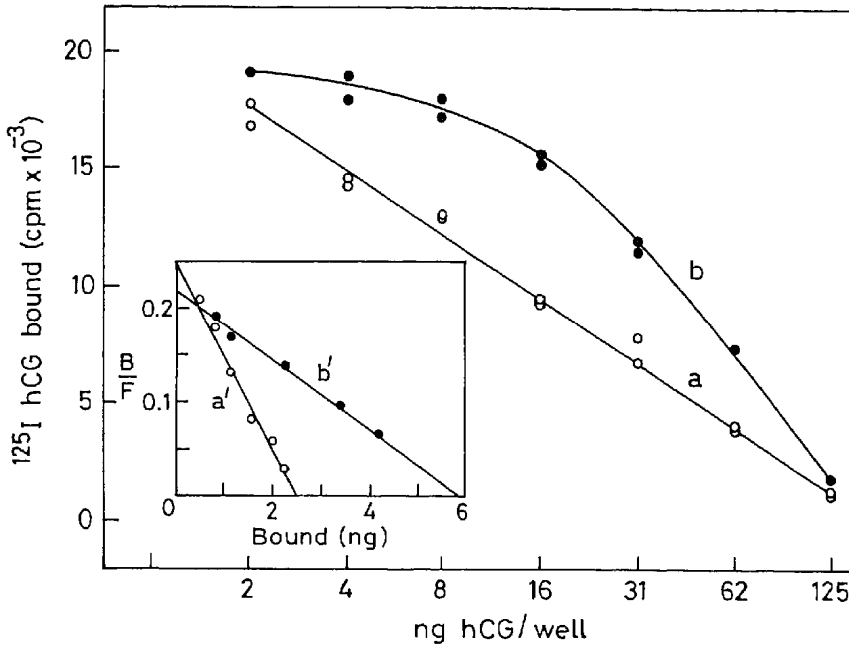


Figure 2. Displacement of [¹²⁵I]hCG from solid phase VM 7 (curve a) and VM 9 (curve b) by hCG in SPRIA. Inset: Scatchard plot analysis of the SPRIAs (curve a' VM 7 and curve b' VM 9 respectively). MAbs were coated at 1/50 dilution in both cases.

Table 1. Comparison of affinity constant measurements by different methods.

Methods/MABs	VM 4	VM 7	VM 9	D 68
Scatchard	170.9	10.0	3.54	18.2
Single point				
20 h binding	200	8.5	2.60	21.3
Single point 20 h	202	20.30	7.20	31.3
binding and		±	±	
dissociation		0.062	0.048	
Specific binding				
(t = 20 h) in cpm	60,000	18,000	16,000	40,000

Affinity constants are expressed as $\times 10^8/M$.

arise because of the heterogeneity of the reaction. This difference is only marginal, considering that dissociation of the complex is not complete and the measured rate constants are only approximations. In several MABs we have observed that as much as 50% of the bound [¹²⁵I]hCG cannot be displaced by 1000-fold excess of hCG even after overnight incubation. K_a obtained by single point dissociation method is shown in table 3. Here effective binding of the antigen is allowed for 5 h (with association times of 14–300 min—see legend for details) and 20 h with a 20h re-equilibration time

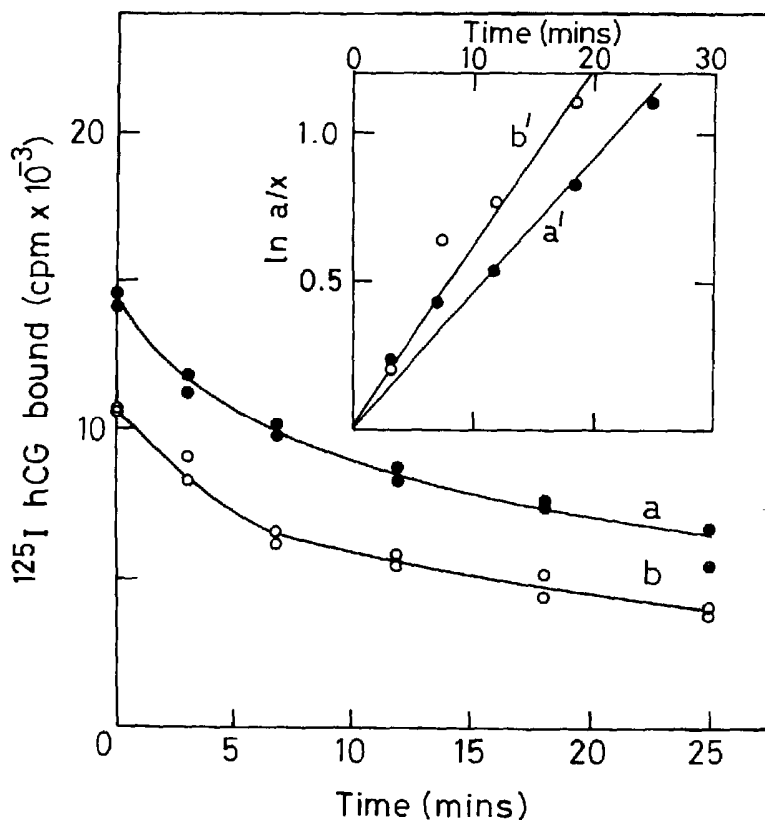


Figure 3. Dissociation of MAb-[¹²⁵I]hCG complex in the presence of 1000-fold excess of unlabelled hCG. The complex was obtained by incubating the solid phase MAb with [¹²⁵I]hCG for 5 h at room temperature. Dissociation was started by adding 250 μ l of 2 μ g/ml hCG to each well. *Inset:* Data fitted into 1st order rate constant (curve a' for VM 7 and curve b' for VM 9). Nonspecific binding (that which could not be displaced even after overnight dissociation) was 2000 cpm and 1500 cpm respectively.

(see footnote under table 3). Apparent K_a is 50% more when initial binding is allowed to occur for 5 h and increases to 100% on increasing the time of initial binding to 20 h for both the MAbs (VM 7 and VM 9).

4. Discussion

Even though ELISA has been used extensively in identifying sequence specific epitopes, its use in determining the reaction characteristics are very minimal. Only recently rate of dissociation of MAb-Ag complex has been investigated (Raman *et al* 1992; Larvor *et al* 1994). Determination of affinity constants using ELISA has been very few and controversial (Underwood 1993). We have utilized in this report SPRIA and SPB to quantitatively measure these parameters and have obtained affinity constants by several independent approaches as described under methods and show that the reaction between solid phase MAb and [¹²⁵I]hCG follows an apparent second order kinetics.

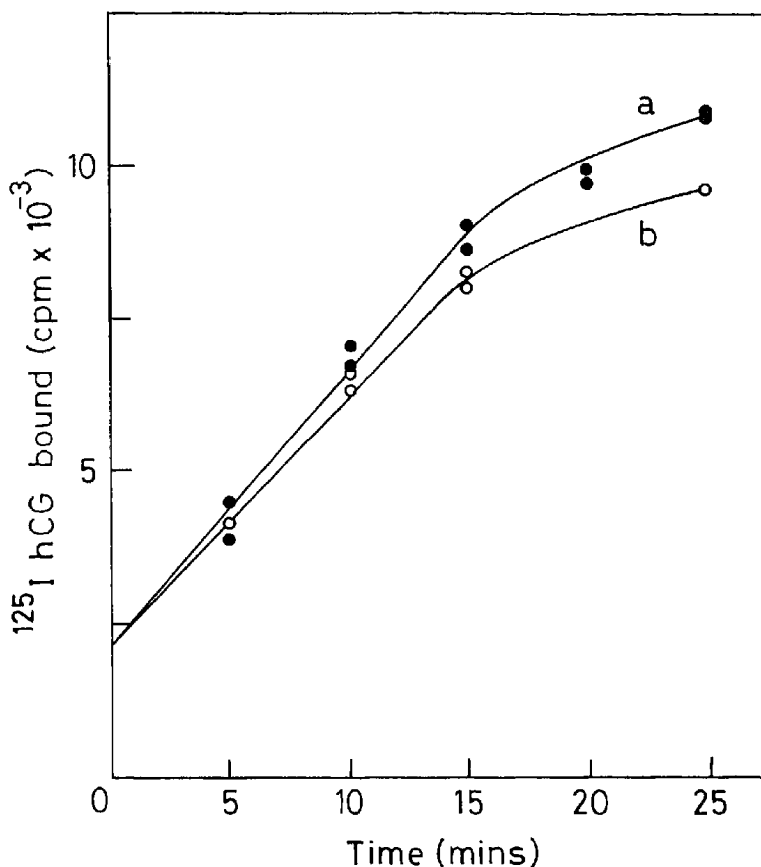


Figure 4. Binding of [¹²⁵I]hCG with time to solid phase VM 7 (curve a) and VM 9 (curve b). Concentrations of MABs were 3.243×10^{-9} (mol/l) and 5.05×10^{-9} (mol/l) respectively.

Data presented above have demonstrated that measurement of kinetic parameters are easily done using SPRIA/SPB. One of the uncertainties that existed in finding the epitope density in a conventional ELISA system has resulted in uncertain affinity measurements (Underwood 1993). Added to this is the observation that several of the MAB-[¹²⁵I]hCG complexes show significant dissociation, as seen in table 1 as well as reported by Murthy and Venkatesh (1996). In the present investigation we have assumed that the interaction of the epitope and paratope is a complete reversible reaction and have treated the results as homogeneous equilibrium system between paratope and epitope, though the reaction is heterogeneous like hormone-receptor reactions. Affinity constants have been measured by several ways as described under methods. Data (table 1) indicate that methods of Scatchard analysis and single point binding agree well and the data obtained by individual rate constant measurements (K_{on}/K_{off}) show 70–80% reduction in the K_a measurements, while overnight binding followed by re-equilibration shows 100% increase. Apparent decrease in K_a with rate measurements can be because of the heterogeneity of the reaction, or may also reflect the non-ideal dissociation (see below) where several MAB-[¹²⁵I]hCG complexes dissociate to a maximum of 50% in the presence of 1000-fold excess of unlabelled hCG. At this point of time it is difficult to say which of the method gives true K_a . But it is clear

Table 2. Comparison of kinetic parameters of epitope-paratope reaction.

MAB	VM 7 ^a	VM 9 ^a
Affinity constant ^b (Scatchard)	7.3×10^8	3.14×10^8
Affinity constant (single point binding method)	$8.26 \pm 0.36 \times 10^8$	$3.19 \pm 0.19 \times 10^8$
Rate of forward reaction K_{on}	$1.4 \times 10^7/\text{M min}$	$0.77 \times 10^7/\text{M min}$
Rate of backward reaction K_{off} (by dissociation data) ^c (1st order rate constant)	0.05/min	0.06/min
K_a by calculation (K_{on}/K_{off})	2.8×10^8	1.3×10^8

^aApparent capacity of the wells as measured by the Scatchard plot in this set of experiments was 3 ng/ml and 5 ng/ml for VM 7 and VM 9 respectively. These values have been used in the determination of the second order rate constant of the forward reaction. Specific activity of [¹²⁵I]hCG was 42,000 cpm/ng of hCG.

^bAffinity constant is expressed in M⁻¹.

^cThe dissociation was not complete even after 20 h at room temperature. Undissociated radioactivity was 2000 cpm and 1500 cpm respectively in VM 7 and VM 9. These were taken as nonspecific binding for calculation of rate constant in the above experiments.

Table 3. Affinity constant using single point dissociation method for [¹²⁵I]hCG bound for 5 h and overnight to solid phase MAb.

Binding time(h)	MAb VM 7			MAb VM 9		
	cpm bound			cpm bound		
	a	b	K_a^*	a	b	K_a^*
5 (14')	8395	2218	1.41	7626	1602	0.41
5 (27')	10624	2623	1.30	9482	1957	0.44
5 (41')	12511	3120	1.32	10597	2307	0.43
5 (59')	14262	3859	1.48	12312	2772	0.45
5 (87')	15210	3977	1.43	13845	3200	0.47
5 (300')**	17403	4796	1.54	15581	3661	0.48
20**	19318	6405	2.04	16600	5212	0.72

a, cpm bound at the start of re-equilibration.

b, cpm still bound after 20 h re-equilibration.

* K_a , Affinity constant measured for each value $\times 10^9$.

**If 480 cpm of 4796 cpm (10%) is considered non dissociable the K_a determined will be 1.3×10^9 . If 1200 cpm of 6405 cpm bound (20%) is considered non-dissociable the K_a determined will be 1.46×10^9 .

14', 27', 59', 87' and 300' represents the time of association of the [¹²⁵I] hCG to MAb. After arresting the association at these time points the wells were left at room temperature before starting the re-equilibration (by adding 250 μ l of RIA buffer/well) at 300 min (5 h) of effective interaction with MAb. Re-equilibration was done for 20h at room temperature.

that values obtained are consistent no matter which method is used, and as long as the same procedure is used the relative values are acceptable for interpretation.

One of the discrepancy which becomes obvious (tables 1 and 3) is that the measured affinity by single tube dissociation is in variance with other methods. The measurements are on the higher side by as much as 50% (for 5 h binding data) to 100% (for 20 h binding data). Table 1 indicates that when binding of [125 I]hCG is high discrepancy is less. Reason for this discrepancy is indicated from dissociation data of VM 7 and VM 9-[125 I]hCG complexes (figure 3). Dissociation of the complex is not complete even after 20 h incubation with 1000-fold excess of hCG. This indicates that in addition to an easily dissociable complex, a non-dissociable complex is also slowly formed, and for complexes of MAbs VM 7 and VM 9 they contribute to about 20%. Thus in the experiment where overnight association occurs significant quantum of the [125 I]hCG has gone to the non-reversible system and hence true equilibration is between the dissociable part of [125 I]hCG and RIA buffer. This brings about an apparent increase in the affinity constant. It is seen more clearly in polyclonal antisera where non-dissociable component of the complex reaches almost 75% (data not shown). Thus for accurate determination of association constant it is preferable to utilize Scatchard plot or single point binding data. Calculation shows that if 25% of the radioactivity that remains bound (corresponds to only 10% of the bound radioactivity at the start of dissociation) is non-dissociable the reduction in K_a measured is 100% arising out of errors in the measurements of K_{off} . However non-reversible binding of this magnitude would hardly matter in the single point binding data. Hence as a rule when single point determinations needs to be done it is necessary to use single point binding data and avoid single point dissociation data, even though the later data is more consistent than the former or Scatchard analysis.

The results also show that forward and backward reaction rates can indeed be determined with relative ease with solid phase MAbs. Comparison of K_{on} determined by biosensor technology using Surface Plasmon Resonance (BIAcore, Pharmacia) and ELISA methods is in the order of $2-50 \times 10^4/s$, which compares with those obtained with this method [$(13-23 \times 10^4/s)$] (Larvor *et al* 1994; Roussel *et al* 1995). K_{off} , reported by ELISA and other methods are in the range of $0.1-8 \times 10^{-3}/s$ (Roussel *et al* 1995), same as those obtained in these studies ($0.8-1.0 \times 10^{-3}/s$). Thus kinetic parameters of epitope-paratope interaction can be investigated using solid phase MAbs and radiolabelled antigen. The method described above can hence be used in understanding epitope-paratope interaction at molecular levels. Determination of the kinetic constants using modified antigens/or antigens obtained by site directed mutagenesis also helps in understanding quantitatively roles of side chain residues in epitope paratope interaction with relative ease. We have seen that modification of a lysine residue in β hCG changed affinity constants for MAb VM 7, which can be investigated by the above methodology to understand the role of side chain residues of antigens in the interaction. In addition to these advantages it is possible to envisage its utility to obtain few thermodynamic parameters of epitope-partope interaction like in BIAcore system (Roussel *et al* 1995) with minimal quantities of antigen and Mabs.

Method described in the above adds to the battery of methods used presently for determination of rate constants, namely fluorescence quenching, ELISA and recently introduced biosensor methods. Each method have their advantage. Among the above methods only fluorescence quenching measures dissociation constant in homogeneous reaction. However, the method is very demanding in terms of the quantity and quality

of the antigen and the antibody. Measurement from other methods represent the dissociation constant in heterogeneous system with either the ligand or the ligate being immobilized. However, dissociation rate constant obtained by ELISA and fluorescence quenching methods have been shown to be comparable. ELISA method though convenient has limitation because of the dissociability of several MAb-Ag complex, and can introduce errors in dissociable MAb-Ag complex studies. Recently introduced biosensor method though much in use is quite expensive and needs sophisticated infrastructure. The present method requires only μg quantities of unfractionated MAb (culture fluid) and radiolabelled antigen and provides comparable results in terms of kinetic parameters of the ligand-ligate interaction as seen above. This approach can also be used to obtain thermodynamic parameters of Ag-MAb reaction and to investigate protein-DNA interaction. In addition to being an inexpensive substitute to the biosensor technology, the method has the potential to be automated to handle large number of samples at the same time. While uncertainties remain about absolute values like in any other heterogeneous methods, it can be used very effectively for relative quantitation of both rate constants to study the Ag-MAb interaction through approaches like thermal and solvent perturbations, chemical modification, etc. Such analyses not only enhance our basic knowledge on the mechanism of these interactions, but also provide additional useful tool in selection of neutralizing MAbs and synthetic immunogens for vaccination. This method can be extended to analyse other biospecific interactions like DNA-protein interaction, DNA-DNA hybridization, protein-peptide interaction, etc. Further the methodology described here serves as an inexpensive alternate to the biosensor technology (BIAcore, Pharmacia) to study ligand-ligate interaction.

Acknowledgements

Authors are grateful to Dr Anjali Karande (BC, IISc) for kind help and suggestions during the course of MAb production. We wish to thank Prof. James Dias, Wadsworth Research Laboratories, New York, USA for MAb 68·K12·1D12. We also thank Prof. M S Shaila (MCBD, IISc) and Prof. A J Rao (CRBME, IISc) for allowing us to use their tissue culture facility. We are grateful to Prof. N R Moudgal for his constant interest and encouragement. NV acknowledges the Council of Scientific and Industrial Research, New Delhi for a senior research fellowship.

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Corresponding editor: INDIRA NATH