Realtime kinetic analysis of antigen–antibody interaction using solid phase binding: Transformation of hCG-monoclonal antibody complex

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Kinetic constants of MAb–hCG interactions have been determined using solid phase binding of ^{125}I[hCG] to immobilized MAb. While association has been shown to follow the expected pattern, dissociation consists of at least two reversible steps, one with a rate constant of 0.0025 min^{-1}, and a second with a rate constant of 0.00023 min^{-1}. Validity of affinity constant measurements in the light of the complex reaction kinetics is discussed. A comparison between the method of surface plasmon resonance technology (BIAcore) and solid phase binding (SPB) for determination of kinetic parameters shows that SPB provides not only a cost-effective approach for determination of realtime kinetic parameters of macromolecular ligand–ligate interaction but also a method with several advantages over the BIAcore system in investigating the mechanism of antigen–antibody interaction.

ANALYSIS of ligand–ligate interaction has found renewed surge of interest with the development of surface plasmon resonance technology to study realtime kinetics of the interaction with BIAcore[1–5]. Using this approach kinetic constants have been determined for several ligand–ligate pair, most common being MAb–antigen pair. Several kinetic and thermodynamic constants obtainable by the BIAcore can also be obtained, in theory, by radiolabelled ligand binding to MAbs in conventional approach like liquid phase RIA (LPRIA), enzyme-linked immunosorbent assay (ELISA), etc. However this simple approach has not been seriously attempted in the past, probably for the following reasons. First, extensive use of ELISA, in most of the MAb-related research, was the method of choice for study. However attempts to use ELISA for determination of even simple equilibrium constant were inconsistent[6–12]. Other alternate method, namely LPRIA with MAbs is cumbersome for kinetic studies with radiolabel being an added deterrent. In addition, as has been shown by us recently, both the methods LPRIA & ELISA are multistep processes and suffer from post-equilibrium disturbance, due to easy dissociability of the MAb–Ag complex[13,14]. Development of single step solid phase assay using immunochemically adsorbed antibodies allows instantaneous termination of the reaction at equilibrium, and hence, eliminates uncertainties that exists in ELISA/LPRIA[15]. Thus binding of radiolabelled antigen to immobilized MAbs provides a method by which kinetic analysis of antigen–antibody interaction can be investigated. In the following we determine the kinetic constants of the reaction and analyse dissociation pattern of MAb–^{125}I[hCG] complex and discuss results in the light of BIAcore analysis.

Materials and methods

Human chorionic gonadotropin (hCG) was prepared from early pregnancy urine and characterized as reported earlier[16]. Iodination grade hCG was obtained from NHPP, Bethesda, USA. MAb VM4 was raised in our laboratory by a standard procedure using hCG $\alpha\beta$ dimer for immunization. This MAb was found to be specific for the native β subunit and reacted well with the dimer.

Iodination of hCG was carried out by iodogen method[17]. Specific activity was calculated by the extent of incorporation. Unless otherwise stated, the specific activity was 45,000 cpm/ng hormone. Freshly prepared ^{125}I[hCG] was used in all kinetic experiments.

Scatchard plot analysis

MAb was immobilized through immunochemical bridge on microtiter wells as already described[13–15]. MAb VM4 culture fluid was coated at a dilution of 1/1000 for SPRIA. SPRIA was carried out by the standard procedure and the data was subjected to Scatchard analysis to obtain apparent affinity constant[18] (K_a).

Determination of association constant

^{125}I[hCG] was added to VM4-coated wells (coated with 250 µl of 1/50 VM4 culture fluid) and binding was ter-

minated at different periods of time by washing the wells with RIA buffer. Radioactivity bound to the wells was monitored and used for determination of the rate constant by the following formula.

$$k_{+1} = [dx/dt]/[Ag] \cdot [MAb],$$

where dx/dt represents the slope of the binding data, [Ag] and [MAb] represent the initial concentrations of antigen and antibody, all expressed in moles/litre.

Determination of dissociation rate constant of hCG-VM4 complex

^{125}I[hCG] was bound to immobilized MAb overnight, washed and counted in a multigamma counter. Dissociation was started immediately by adding 250 µl of hCG (2 µg/ml) in 0.05 M phosphate buffer, pH 7.0

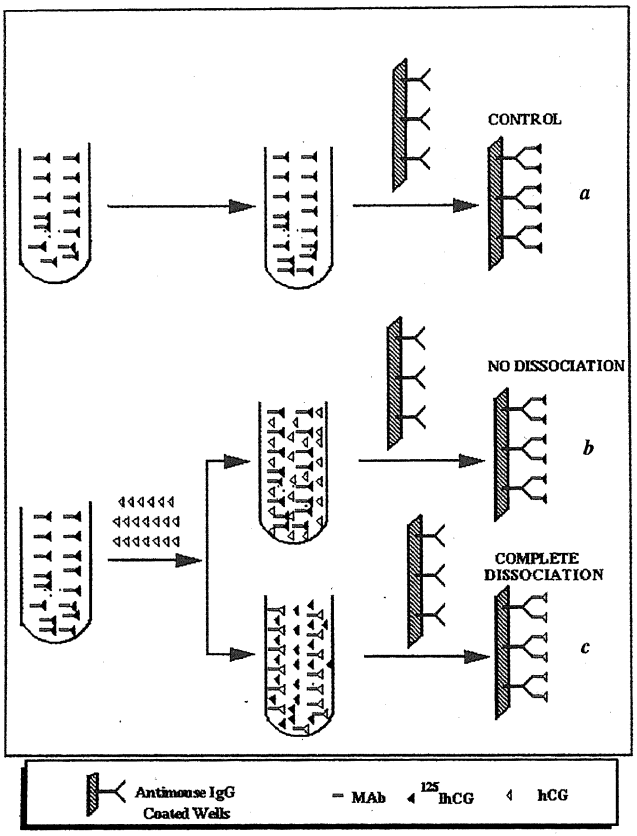

Figure 1 *a–c*. Schematic representation of the dissociability of ^{125}I[hCG]-VM4 complex in liquid phase (*a*) control. (*b*) and (*c*) show the situation when the ^{125}I[hCG]-MAb complex is completely non-dissociable or completely dissociable. In a completely dissociable situation, the binding of the complex formed in the liquid phase to the Mouse DAb coated well will be nonspecific (*c*), while in the case of complete nonreversibility it will be like specific binding (*b*) as in the control (*a*). Intermediate values present partial reversibility (See methods for experimental details).

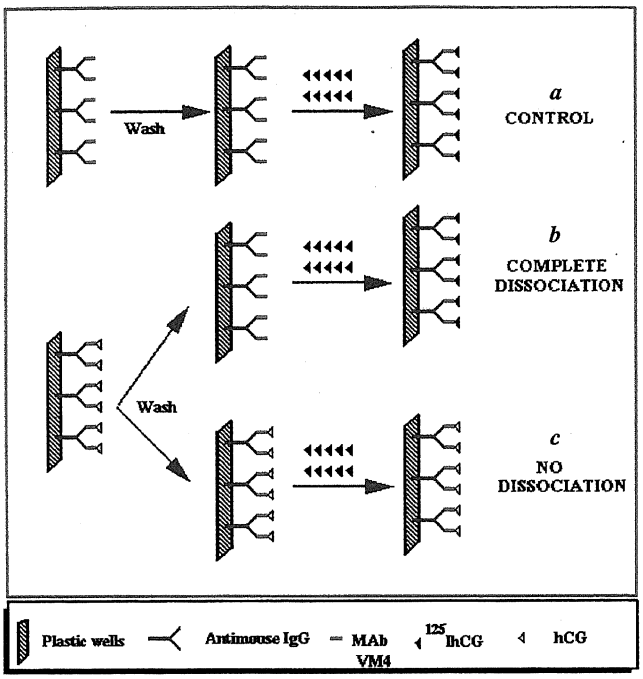

Figure 2 *a–c*. Schematic representation of the dissociability of hCG–VM4 complex from the solid phase (*a*), binding of ^{125}I[hCG] to VM4 coated well (control). (*b*) and (*c*) show sequence of events when the VM4–hCG complex dissociates completely or do not dissociate. Complete dissociation results in binding of ^{125}I[hCG] comparable to control (*a*) and lack of dissociation results in nonspecific binding (*c*). Intermediate values present partial dissociation (See methods for experimental details).

containing 2% bovine serum (RIA buffer). At known intervals of time, the supernatant was discarded and the radioactivity bound was measured in the same carrier of the multigamma counter as done before (this is to take care of different counting efficiencies of the carriers). Radioactivity measured at each point of time is expressed as percentage of the radioactivity retained by the well[19]. For determining the dissociation rate constants plot of $\ln[(a-b)/(x-b)]$ vs time was drawn from the data. Value of a was 90%, while that of b was 48% for fast dissociation rate constant (x was taken between 0 and 400 min for the fast dissociation), while for the slow dissociation they were 48% and 0% respectively (x values taken for 13 h, 24 h and 48 h: see under results for details).

Capacity measurement of the well

Capacity of the well to bind hCG was measured by specific binding, specific activity of the ^{125}I[hCG] and K_a determined by Scatchard plot using the formula

$$C_{MAb} = [B/F + K_a \cdot B] \cdot [1/K_a],$$

where B and F represent bound and free antigen concentrations, C_{MAb} represents the capacity of the well and K_a affinity constant. This method is routinely used for determination of the concentration of MAb required for rate constant measurements.

Determination of apparent nonreversibility

In liquid phase. The rationale of the approach is shown in Figure 1. Experimentally, ^{125}I[hCG] (200,000 cpm) was incubated with VM4 antisera in the liquid phase in a tube for 1–2 h at room temperature. 100 µl of the ^{125}I[hCG]–VM4 complex (100,000 cpm) is added to immobilized antimouse IgG (in microtiter wells) and binding carried out for 2–4 h at room temperature. As control, the same amount of ^{125}I[hCG] (100,000 cpm) incubated in RIA buffer was added. Radioactivity bound to the wells is a measure of the extent of nonreversibility.

In solid phase. Figure 2 schematically represents the methodology of the experiment (see legend for details). Immobilized VM4 on plastic wells was saturated with hCG by incubating with 2 µg/ml hCG for 20 h. Unbound hCG was washed off and hCG bound to the antibody was incubated with RIA buffer (250 µl) for 2 h to allow dissociation to occur. The wells were washed, further dissociation allowed to occur for an additional ten times. Finally ^{125}I[hCG] (100,000 cpm, 250 µl) was added to the well and after 20 h incubation period the radioactivity bound was measured in a LKB multigamma counter. As control VM4 adsorbed wells were also washed with RIA buffer the same number of times, except they were was not saturated with hCG. Difference between the specific binding of these two sets gives an index of the extent of nonreversibility.

Determination of affinity constant

By Scatchard plot. Affinity constant was obtained by the standard method already described.

From re-equilibrium data. ^{125}I[hCG] (250 µl, 100,000 cpm) was added to immobilized VM4 and specific binding after 20 h incubation was determined (S). To each of the wells was added 250 µl of RIA buffer and re-equilibration of the ^{125}I[hCG] allowed to occur between the solid phase and solution phase for 20 h at room temperature, supernatant discarded and radioactivity bound was measured (B). Another set of wells were incubated overnight with 250 µl of 2 µg/ml hCG. Radioactivity that remained bound to this was taken as 'apparently nondissociable' ^{125}I[hCG] from the complex (N). From this data K_a was calculated by the formula

$$K_a = [B - N]/[S - B] \cdot [(C_{MAb} - N) - (S - B)],$$

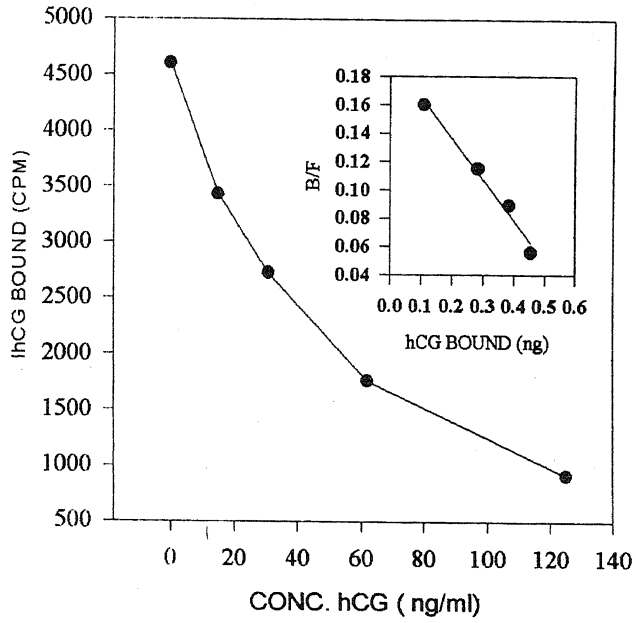

Figure 3. SPRIA and K_a determination by Scatchard and single point binding. Inset shows Scatchard plot for the displacement analysis.

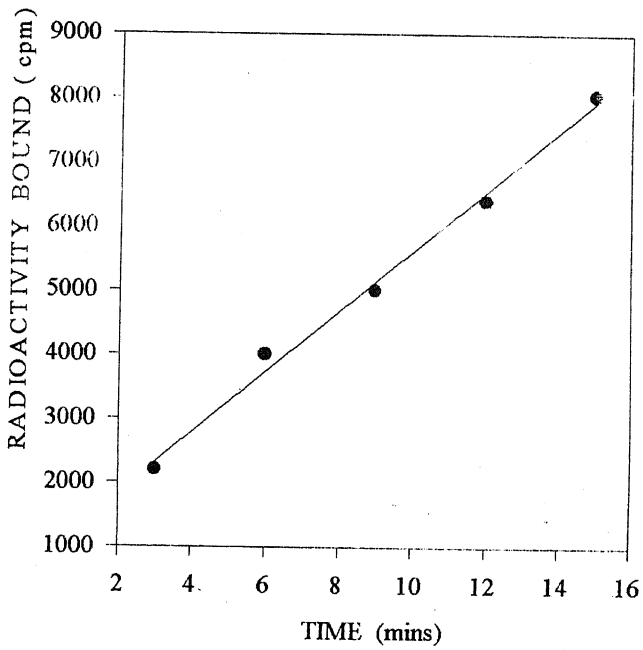

Figure 4. Determination of k_{+1} by binding data: ^{125}I[hCG] = 112,000 cpm/well of specific activity 26,000 cpm/ng added to VM4 coated wells at 0 time. Capacity of the MAb adsorbed calculated from overnight binding was 5.193×10^{-10} M and that of ^{125}I[hCG] 4.657×10^{-10} M respectively (see for details under methods). Affinity constant taken for calculation is 3×10^9/M, obtained from the data of Figure 3.

where all values are expressed in concentrations (moles/litre). C_{MAb} is the concentration of the MAb immobilized on the well, obtained from overnight binding data as explained earlier.

From rate constants. Forward rate constant (k_{+1}) and reverse rate constant/s (k_{-1}) determined as described above have been used to derive K_a by the formula

$$K_a = k_{+1}/k_{-1}.$$

Results

Scatchard plot analysis of SPRIA using immobilized VM4 for the determination of apparent affinity constant gave a value of 3×10^9 M^{-1} (Figure 3). Affinity constant measured by single well binding also gives comparable results[19]. Hence in all further experiments concentration of bound MAb is calculated from single point binding data as explained under methods.

Binding of ^{125}I[hCG] to immobilized VM4 with time is linear (Figure 4). Rate constant of association measured using the slope of this plot is 8×10^6 M min^{-1}. This is comparable to that obtained in several MAb–Ag pair using the BIAcore method[20–22].

In contrast to the association profile which follows a linear pattern, dissociation profile of the ^{125}I[hCG]–MAb is more complex. Even after 8 h of incubation with excess unlabelled hCG complete dissociation of the complex does not occur (Figure 5) with further dissociation occurring rather slow but certain (inset), indicating two distinct rates of dissociation, and the two rate constants of dissociation are 0.0025 min^{-1} and 0.00023 min^{-1} respectively (Figure 6). The first rate constant (k_{-1}) is obtained by assuming the total dissociable ^{125}I[hCG] to be 52% based on the extrapolated

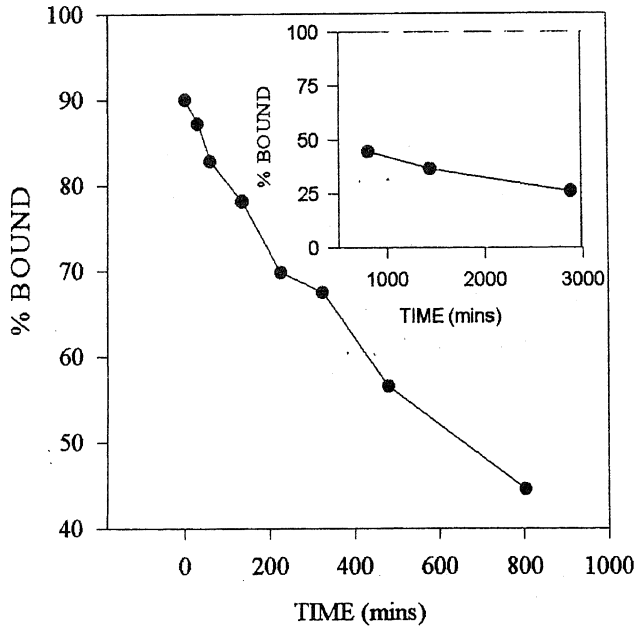

Figure 5. Dissociation profile of bound ^{125}I[hCG]–MAb complex: ^{125}I[hCG] (86.000 cpm of specific activity 42,000 cpm/ng, 250 µl) was added to microtiter wells previously coated with MAb VM4 at 1/100 dilution for overnight binding (cpm bound = 35876 ± 2918), and dissociation started by adding 250 µl of unlabelled hCG (200 ng/well), Inset shows the extent of retention of binding for extended periods of incubation (13 h to 48 h).

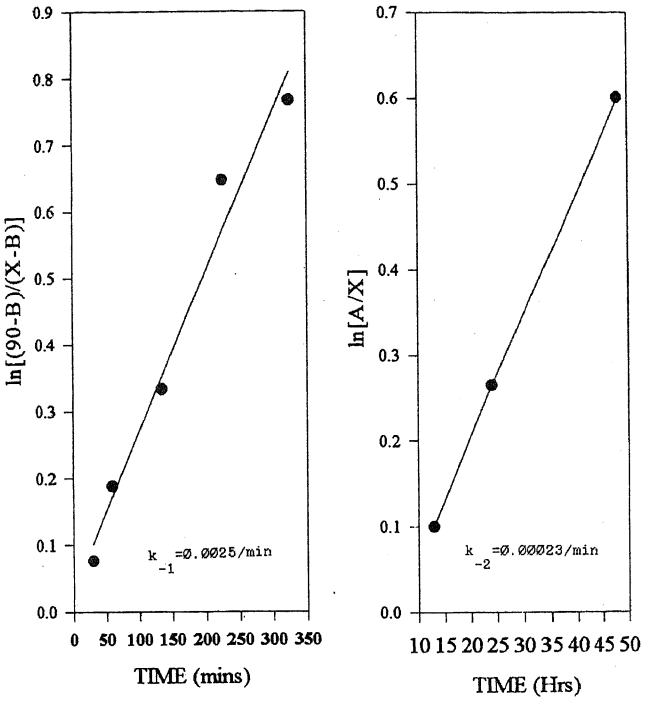

Figure 6. Plot for determination of dissociation rate constant. Extrapolated binding to 0 time is considered (52%) as maximum dissociable for the determination of the fast rate of dissociation ($a = 90$, $b = 48$). Right panel shows the 'dissociation rate constant' obtained for the slow dissociation with $a = 52$, and $b = 0$ (See under methods for details).

Table 1. Dissociation of the ^{125}I[hCG]–VM4 complex in liquid phase binding (^{125}I[hCG] bound to VM4 coated wells in cpm)

VM4 dilution used during complex prepn	Non sp.* control	Exptal	Sp. control	% non-reversible
1/400	1845	12,300	33,212	37
1/800	1300	12,500	28,500	43
1/1600	908	8,150	19,800	40
1/3200	520	4,700	12,800	37

*This control presents the ^{125}I[hCG] incubated with VM4 in the presence of 2 µg/ml hCG. Exptal represents ^{125}I[hCG] incubated with VM4 in absence of unlabelled hCG (overnight) to which 2 µg/ml hCG was added and reequilibrated (120 min) before adding to immobilized DAb. Sp. controls represent binding of ^{125}IhCG. VM4 at respective concentrations to immobilized Dab (Antimouse Ig6).

Table 2. Dissociability of hCG-VM4 in solid phase

	Binding of radioactivity to immobilized VM4 (n = 7) cpm ± SD
Control [Binding of ^{125}I[hCG] to immobilized VM4 well]	50,400 ± 3650
Experimental [Binding of ^{125}I[hCG] to immobilized VM4, saturated with hCG and extensively washed for complete dissociation]	33,519 ± 3000

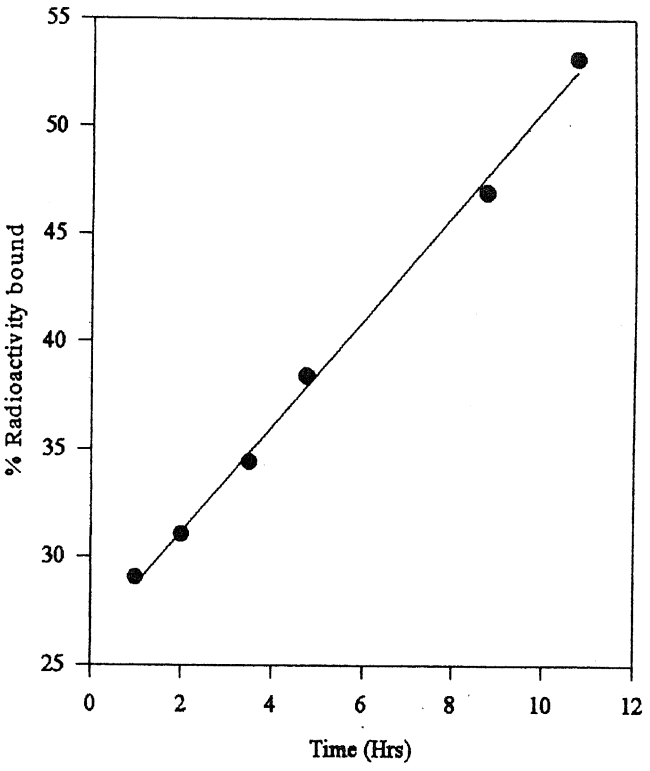

Figure 7. Increase of slowly dissociable portion (transformed complex) of preformed complex of VM4–^{125}I[hCG]. Radioactivity bound at 1 h incubation period (0 time of dissociation) was 16,450 ± 540 (SD) cpm. Unbound ^{125}IhCG was discarded and dissociation started at time periods as indicated in X-axis.

slow dissociation data (curve in the inset of Figure 5 extrapolated to 0 time) while the second rate constant (k_{-2}) is obtained by assuming that the slow dissociation is complete in due course, and analysing the points above 8 h by which time most of the fast dissociation is complete.

Dissociation observed in the above solid phase method is not an artifact arising out of iodination of hCG or because of solid phase binding has been proved by demonstrating that reaction of the MAb with ^{125}I[hCG] in liquid phase is as much 'nondissociable' as in the solid phase (Table 1). The extent of slow dissociability seen is about 40%, comparable to that obtained in SPB (35%). Similarly VM4 saturated with unlabelled hCG in the solid phase does not completely dissociate in spite of a dozen washings (Table 2) and rules out slow dissociation as an artifact arising out of labelled hCG. Apparent nondissociable portion of the binding increases linearly with time (Figure 7) in absence of either of the reactants. This increase is quite significant going up to 50% in 8 h. However incubation of this complex with unlabelled hCG slowly releases the ^{125}I[hCG] into medium, indicating this apparent nondissociation to be infact a slow dissociation. The result also indicates that this transformation appears to be spontaneous, as it occurs in the absence of any other reactants.

On the basis of these kinetic evidences, the mechanism of MAb–hCG interaction can be presented as follows to account for the rate constants.

$$\mathrm{Ag} + \mathrm{Ab} \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} \mathrm{Ag\text{–}Ab} \underset{k_{-2}}{\leftrightarrow} \mathrm{Ag\text{–}Ab^*}, \qquad (1)$$

where Ag–Ab and Ag–Ab* present dissociable and transformed complex respectively. Conversion (apparent dissociation) rate constant of Ag–Ab* to Ag–Ab is about 10% compared to the dissociation rate of Ag–Ab to Ag and Ab. This proposed mechanism is for MAb VM4, but is likely to be true for other epitope–paratope pairs. Relevance of this slowly transformed Ag–Ab* in physiology also needs to be studied further. Apparent nonreversibility of several polyclonal antibodies clearly indicates that such transformed species are present in polyclonal antisera–antigen complexes and hence may have relevance in physiological systems.

On the basis of these studies, there are several ways to obtain affinity constants of VM4–^{125}I[hCG] interaction. It is certain that K_a as determined by Scatchard plot, though extensively used, may not quantify avidity of the system. A comparison of affinity constants measured for this system using different methods shown in Table 3 demonstrates that the affinity constants measured are not very different from Scatchard plot data. However caution should be exercised in the choice of the disso-

Table 3. Affinity constant measurements by different methods (M^{-1})

Scatchard plot from SPRIA	3.00×10^9
By single well binding	2.97×10^9 (n = 6)
By reequilibration data	6.60×10^9*
By k_{+1}/k_{-1} (using fast dissociation)	3.33×10^9**
By k_{+1}/k_{-1} (using slow transformation)	33.30×10^9***

*Input ^{125}I[hCG] = 86,000 cpm, bound overnight = 59,000 cpm, nondissociable by 48 h incubation with hCG = 9500 cpm, ^{125}I[hCG] bound after overnight reequilibration in 250 µl of RIA buffer = 29,200 cpm.
**Using k_{+1} of 0.8×10^7 min^{-1} and k_{-1} of 0.0025 min^{-1}.
***Using k_{+1} of 0.8×10^7 min^{-1} and k_{-2} of 0.00023 min^{-1}.

ciation rate for the calculation of the K_a, because slow transformation is an integral part of the binding reaction.

Discussion

Reaction kinetics have been investigated in several systems using radioligands. Unsuccessful attempts of the same approach with antigen–antibody interaction can be traced to the error of the analytical tool that was adopted, namely ELISA and LPRIA. Both are multistep assay systems, and during post-equilibration period reversibility of MAb–^{125}I[hCG] has been clearly demonstrated to introduce errors in interpretation[13]. Solid phase binding (SPB), a single step method, which eliminates the errors arising out of reversibility has made it possible to investigate the kinetic parameters.

Results presented clearly demonstrate that kinetic constants can be measured using binding of radiolabelled ligand to MAb adsorbed immunochemically to plastic wells. Binding of ^{125}I[hCG] to immobilized matrix follows a second order reaction as expected with a measurable rate constant which falls in the range of the data generated for several MAb–Ag pairs using the BIAcore system. Contrary to expectations, dissociation in presence of 1000-fold excess of hCG is not complete though it follows a first order reaction with measurable rate constant, and 35% of the radiolabel remains bound to the well even after 20 h of incubation. Also, this apparent nondisplaceability is not artifactual arising out of iodination of the hCG, as nondissociability is seen in solid phase binding of unlabelled hCG (Table 2). In fact immobilized MAb–hCG (saturated with unlabelled hCG) after repeated washing fails to bind ^{125}I[hCG] to the same extent as free immobilized MAb (Table 2), and clearly demonstrates that partial nonreversibility is indeed true in both solid and liquid phases even when using nonradioactive ligand. Though the extent of nondissociability is 30% with VM4, several antibodies show as much as 50–80% nondissociability, and hence indicate its significance.

Based on the two step pattern of dissociation, a reversible one and the second slow-reversible one, reaction of an MAb with ^{125}I[hCG] can be presented as in (eq. 1). First step promoted by epitope–paratope interaction leads to a complex (Ag–Ab), which is in reversible equilibrium with free antigen and antibody; second step consists of its transformation to another complex (Ag–Ab*). One of the immediate questions this complex dissociation data projects is the validity of affinity constants extensively employed as a quantitative measure of avidity. Affinity constants measured by different methods tabulated show that it is not significantly different. Scatchard value is similar to that obtained by individual rate constant determinations representing the first step of the reaction. However affinity constants as measured from re-equilibrium results (Table 3) are also comparable though on the higher side. Considering that in this case the extent of nonreversibility is 30%, K_a measured by all the methods are comparable. If nondissociability were to be 50–80% as it happens in some MAbs, the measured K_a considering the nondissociable portion could be different from that obtained by direct Scatchard measurements/binding data/$[k_{+1}/k_{-1}]$ measurements. The nondissociability data indicate that caution is needed in assessing K_a by Scatchard plot, and in adopting appropriate methods for relative measurements. An absolute measure of K_a is unlikely to be determined from binding data methods, like Scatchrd plot, single well binding, etc. Infact affinity constant needs to be redefined to the requisite step of the reaction.

Analysis of the dissociation data points to transformation of the primary Ag–Ab complex to a transformed (slow-dissociable) Ag–Ab* type (Figures 6, 7). The chemical or physicochemical events which lead to this are not known presently. This is not associated with a covalent binding of MAb–hCG is certain as 5% formic acid completely dissociates the radiolabelled hCG instantaneously (data not shown). How the transformation is related to epitope–paratope interaction is also unclear. Likewise physiological significance of the transformed complex is unknown.

Analysis of the kinetics of dissociation shows that the reaction of an antigen with its antibody is not a simple epitope–paratope reaction and the mechanism proposed here is a minimal requirement. While kinetically the mechanism of the reaction can be presented as shown in eq. (1), additional chemical data is needed to investigate the nature of the reaction. But, it is certain that the interaction of antigen–antibody though promoted by epitope–paratope pair may have other components which are unknown. The regions involved in subsequent conversions are not known, but definitely it is not promoted by the presence of either hCG or Mab (Figure 7).

There are two other methods reported which can measure kinetic constants of MAb–Ag reaction – the BIAcore method which uses surface plasmon resonance[1–5,20–23], and the method of Larvor *et al.* using liquid phase reaction coupled to ELISA for determination of dissociation rate constant[24]. Presently, the former method is used widely because of the ease of determination and the rapidity with which results can be obtained. The method used in this paper (SPB method) is comparable to the BIAcore method in that both are heterogeneous reaction systems where one of the ligands is immobilized. Moreover, association and dissociation constants can be determined by both the methods. Comparisons between these two systems are illustrated in Figure 8. The top panel shows diagramatically the flow-cell of a BIAcore having dimensions of $50 \times 500 \times 2000$ μm. Flow of solutions is through the

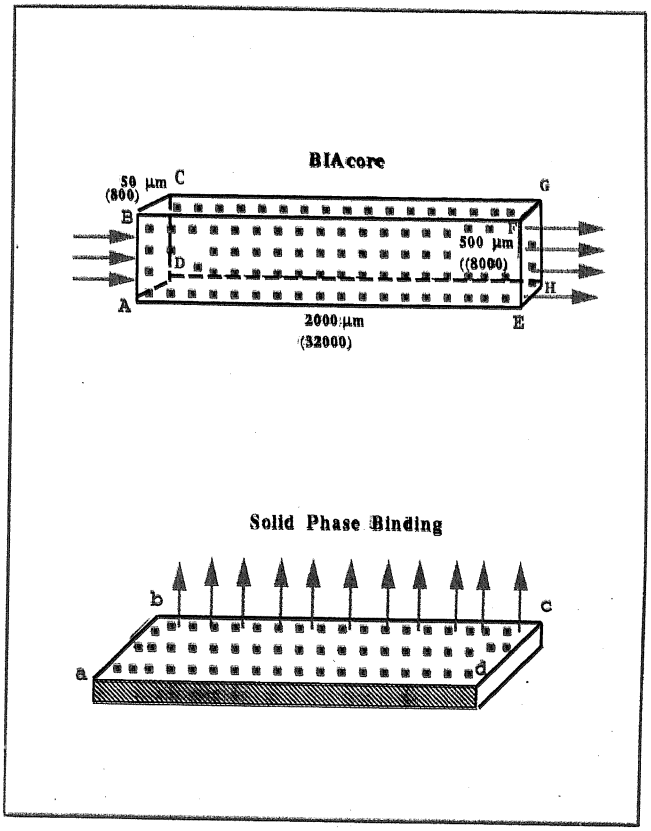

Figure 8. Schematic comparison of the flow cell of BIAcore (top panel) and plastic surface of SPB (lower panel). Arrows show the direction of flow of the solvent in flowcell of BIAcore. ■ presents immobilized ligate covalently bound to the flow cell (in BIAcore) or adsorbed immunochemically (in SPB). Numbers (in μm) represent the dimension of the flow cell, while the corresponding numbers in the brackets represent the number of molecules of the ligate bound along the corresponding axis. Total number of molecules bound in the BIAcore flow cell is about 20×10^{10} molecules. Note that the ligates are stacked up within the flow cell in three dimension, while in the SPB (lower panel) it is distributed in two dimension only.

Table 4. Molecular distance between immobilized antibody molecules in BIAcore cell and in plastic well

Adsorption on	Capacity measured	Surface/ volume	Distance between two molecules
Microtiterwells	4 ng/well	81 sq. mm	6.5×10^{-2} μm
	1 ng/well	81 sq. mm	8.0×10^{-2} μm
BIAcore chip at			
(a) RU of 693	0.5 μM*	60 nl	14.0×10^{-2} μm
(b) RU of 10,000	7.2 μM	60 nl	5.5×10^{-2} μm

*Taken from Hall *et al.*[26].

cross-section of ABCD shown by the arrow. The flowcell has immobilized ligate in space in multilayers illustrated by small squares. Distance between each ligate in both BIAcore and SPB (bottom panel) is about 6×10^{-2} μm, almost 10–50 molecular distances apart from each other (Table 4). Thus both the systems have the same molecular disposition of the ligates, and are subject to similar criticisms on these grounds. While immobilization is done through covalent binding in BIAcore, immunochemical bridge has been used in the SPB method. By the nature of the immobilization of ligates each method has its advantages. Two assumptions made in analysis of the results from BIAcore have been that association is not diffusion controlled and dissociation is not complicated by rebinding of the dissociated ligand. Both the assumptions have been questioned[25,26] and the nature of the problem can be illustrated with Figure 8. In dissociation, which starts with saturated ligand, the solute enters the cells at the cross-section ABCD traverses through and exits at EFGH cross-section. As can be seen in the Figure the dissociated ligand at the cross-section near ABCD has to pass through a very large number of molecules of the immobilized ligate (in this case about 17×10^{10} molecules) which allows rebinding of the dissociated ligand. This error is rather small in early stages of elution (5–10 s by which time about 10 volumes of the solvent has flown through), but tends to become more and more as dissociation increases, effectively distorting the dissociation pattern much like in chromatography and underestimates the true dissociation rate. This effect has been demonstrated by Nieba *et al.*[25], and becomes highly significant if the association constant is of the order of 10^5 M s^{-1}, a range common in most MAb–Ag systems[20–23]. Similarly if the association constant of the ligand is very high [$>10^7$ M s^{-1}] binding is limited by diffusion rather than the binding constant. This is especially so because the ligate is embedded in dextran matrix in three dimension and hence diffusion is an obligatory first step in the binding. Methods have been recently suggested to overcome these problems, but the corrections to be adopted require good knowledge of kinetics, and are unlikely to be the same for all the ligand–ligate pair. In contrast to BIAcore, SPB method utilizes the ligate immobilized on a single surface (Figure 8, lower panel) and does not suffer from the disadvantage of reassociation. Since the ligate is fixed as a monolayer, free access of the ligand to the ligate exists, and hence does not impose a limitation based on diffusion as in the BIAcore. Thus these two major problems associated with BIAcore do not exist with the SPB. Complex dissociability pattern, which has not been quantitated so far in the BIAcore, can be quantitated by the SPB method. Apparent nonreversibility present in protein–protein binding is clearly demonstrated in the dissociation patterns in the BIAcore approach as well, but mostly neglected in all interpretations[20–22]. Quantitation of slow dissociation throws light on the mechanism of ligate–ligand interaction better through kinetic approach. In contrast to these advantages of the SPB

method, need of radiolabelled ligand is a disadvantage needing pure antigen for investigations and a need to prove that the results obtained are not artifactual arising out of iodination. However the results presented here indicate that they are not artifactual and are likely to be the same for all other ligand–ligates. Quantum of the ligate that can be incorporated into the flowcell of BIAcore is 1000-fold more than in the SPB method, which makes it possible to investigate low affinity binding [$\times 10^5$/M] using BIAcore while the affinity of the ligand–ligate should be high [$\times 10^7$/M] for the SPB method.

Though data presented here are for the antigen–antibody pair, the approach is not limited to only this ligand–ligate pair, or to only radioiodine-labelled antigens. The same approach can be used to study other important ligand–ligate pair like that of protein–RNA, protein–DNA, etc. Demonstration of transformation of reversible Ag–Ab complex to slow–dissociable complex (Ag–Ab* type) indicates the possibility that such as yet unrecognized interactions may also be present in other ligand–ligate pair. Thus the SPB method provides a very cost-effective approach to study the realtime kinetics of ligand–ligate interaction. Such studies may be expected to provide new valuable information on the interaction of macromolecular ligand–ligate pair. Importance of the transformed complexes in physiology/physiological processes needs to be investigated.

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