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Analysis of Bispecific Monoclonal Antibody Binding to Immobilized Antigens Using an Optical Biosensor

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Abstract—The interaction between two different monoclonal antibodies (Mabs) and their corresponding bispecific antibodies (Babs) with immobilized antigens was investigated using an optical biosensor (IAsys). The analyzed panel of affinity-purified antibodies included two parental Mabs (one of which was specific to human IgG (hIgG), and another one to horseradish peroxidase (HRP)), as well as Babs derived thereof (anti-hIgG/HRP). Babs resulting from the fusion of parental hybridomas bear two antigen-binding sites toward two different antigens and thus may interact with immobilized antigen through only one antigen-binding site (monovalently). Using an IAsys biosensor this study shows that the bivalent binding of Mabs predominates over the monovalent binding with immobilized HRP, whereas anti-hIgG parental Mabs were bound monovalently to the immobilized hIgG. The observed equilibrium association constant (K_{ass}) values obtained in our last work [1] by solid-phase radioimmunoassay are consistent with those constants obtained by IAsys. The K_{ass} of anti-HRP Mabs was about 50 times higher than that of anti-HRP shoulder of Babs. The dissociation rate constant (k_{diss}) for anti-HRP shoulder of Babs was 21 times higher than k_{diss} for anti-HRP Mabs. The comparison of the kinetic parameters for bivalent anti-HRP Mabs and Babs derived from anti-Mb/HRP and anti-hIgG/HRP, allowed to calculate that 95% of bound anti-HRP Mabs are bivalently linked with immobilized HRP, whereas only 5% of bound anti-HRP Mabs are monovalently linked. In general, the data obtained indicate that Babs bearing an enzyme-binding site may not be efficiently used instead of traditional antibody–enzyme conjugates in the case of binding of bivalent Mabs.

Key words: bispecific antibodies, kinetic constants, bivalent interaction, antigen–antibody interaction, biosensor, IAsys, affinity, avidity

When monoclonal antibodies (Mabs) are used in enzyme immunoassays or other solid-phase systems, quantitative estimation of antibody binding with immobilized antigen is necessary. Two parameters have special importance for Mab binding assessment: the true affinity, characterizing the affinity level of a particular antigen binding site of an antibody towards the given antigenic epitope, and also the avidity or general efficiency of antibody binding to an antigen. The difference between affinity and avidity can be encountered for the ability of bivalent antibody molecules (e.g., IgG class) to interact with an antigen immobilized on the solid phase by two antigen-binding sites simultaneously. In particular, it has been shown that the significant increase in binding force between antibody and immobilized antigen molecules may occur due to bivalent binding [2].

Mabs avidity may have much greater significance for practical applications than the true antibody affinity. Particularly, this should be taken into account for the

Abbreviations: Mabs) monoclonal antibodies; Babs) bispecific monoclonal antibodies; HRP) horseradish peroxidase; hIgG) human immunoglobulin G; K_{ass}) observed equilibrium association constant; k_{ass}) observed kinetic association constant; k_{diss}) observed kinetic dissociation constant.

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design and practical application of so-called bispecific monoclonal antibodies (Babs). Bispecific antibodies can be obtained either by biological techniques, the fusion of two different hybridomas (Babs), or by chemical or gene engineering techniques [3-5]. The unique feature of bispecific antibodies is the presence of two different antigen-binding sites. Babs with an enzyme binding shoulder (e.g., HRP) can be used in immunochemical analysis instead of traditional antibody–enzyme conjugates [5]. It was stated that Babs might have greater advantages in comparison to usual Mabs in various application areas [3-5]. However, it is generally overlooked that in contrast to usual Mabs, the Babs IgGs are monovalent molecules toward each of the antigens. Hence, the reasonable expectation is that the monovalent Babs may considerably lose against bivalent Mabs in terms of avidity of immobilized antigen binding.

The comparison of true affinity between Babs obtained by cell fusion and bivalent Mabs (secretion products of parental hybridomas) in solution has been performed by a number of researchers [6, 7]. Usually, the complete identity of the antigen-binding sites in Babs and Mabs was observed. However, the quantitative comparison between Babs (tetrad secretion products) and bivalent Mabs for their ability to bind the immobilized antigen was performed by only one research group [8, 9]. However, the detailed evaluation of this issue is not only very important for the application of these biomolecules, but also may present a certain theoretical interest for the investigation of interaction mechanism between Mabs and immobilized antigens. It should be emphasized that to date only Fab-fragments were used for the study of binding of Mabs with immobilized antigens as a tool for quantitative evaluation of the true affinity. The bivalent and monovalent interaction was examined based on the differences in antigen binding curves of bivalent Mabs and monovalent Fab-fragments [2, 10-13]. According to the quantitative evaluation of true Babs affinity compared to Fab-fragments, Babs have an advantage of retaining the native structure of the IgG molecule.

This work employs a resonant mirror biosensor (IAsys), allowing monitoring of antigen–antibody binding interactions at the solid phase boundary in real time, without use of labeled reagents [14-16]. The noticeable advantage of this type of biosensors for the investigation of antibody binding to the immobilized antigens is the possibility of express determination of not only the observed equilibrium association constant (K_{ass}), but also the observed association and dissociation rate constants (k_{ass} and k_{diss}) [17-19]. Data handling was performed by specialized software (FASTfit).

The presented work is aimed at quantitative comparison of bivalent binding of Mabs and Babs with immobilized antigens, horseradish peroxidase (HRP) and human IgG1 (hIgG1), using an optical biosensor IAsys. As shown earlier, the true affinity of Mabs is identical to the corresponding antigen binding site of Babs [7]. The kinetic constants for antibody association and dissociation with immobilized hIgG1 and HRP were measured using the biosensor. The analysis of the binding of Babs and Mabs presented in this study allowed to deduce the ratio for monovalently and bivalently bound Mabs associated with immobilized antigens.

MATERIALS AND METHODS

1. Characterization of parental Mabs and Babs. Antibodies were analyzed towards two antigens of different molecular weight and structure, HRP ($M_r \approx 40{,}000$) and hIgG ($M_r \approx 160{,}000$). The cell lines of hybridomas and tetradomas obtained previously [7] were used as the antibody sources in this study. Parental hybridoma 36F9 produced HRP-specific Mabs (isotype IgG1). Parental hybridoma 75G5 produced Mabs (isotype IgG1) specific towards all sub-classes of human IgG. Tetradoma 36F9×75G5 produced Babs (isotype IgG1/IgG1) specific towards HRP and all sub-classes of human IgG (anti-HRP/hIgG). Hence, Babs not only comprised antigen-binding sites identical to parental Mabs, but also had the same structure of the constant part as the parental Mabs [7].

2. Purification of monoclonal antibodies. Anti-HRP Mabs (clone 36F9) and anti-hIgG Mabs (clone 75G5) were purified by affinity chromatography using HRP-Sepharose and IgG-Sepharose (accordingly) from the ascites obtained after mouse inoculation by the corresponding hybridoma cells. Preparation of affinity carriers, affinity chromatography, and buffer systems were described earlier in detail [20]. The purity of antibody preparations was monitored using SDS-PAGE in 12.5% polyacrylamide gel [21]. The control experiments show that more than 99% of affinity-purified antibodies retain their immunological activity, because under the repeated affinity chromatography procedure the antibodies are capable of rebinding to the affinity carrier (data not presented).

3. Determination of the protein concentration. Protein concentration was determined using an optical technique, assuming that $A^{1\,cm}_{280\,nm} = 14$ corresponds to the purified antibody solution with a concentration of 10 mg/ml [22], while $A^{1\,cm}_{403\,nm} = 22.75$ and $A^{1\,cm}_{280\,nm} = 7.3$ correspond to a 10 mg/ml solution of purified HRP [23].

4. Determination of antigen-binding activity of antibodies after affinity purification. HRP (Calbiochem, USA, $R_z > 3.0$) or hIgG1 (kindly provided by T. N. Batalova, Gabrichevsky Institute of Epidemiology, Moscow) were incubated on the immunoplates (Medpolimer, Russia) overnight at room temperature (10 μg/ml in 0.05 M sodium-carbonate buffer, pH 9.5; 100 μl per well). Antibody dilutions were prepared in the concentration range of 1-1280 ng/ml. Antibodies were

dissolved in 0.025 M sodium-phosphate buffer, pH 7.4, containing 0.15 M NaCl, 0.5 ml/liter of Tween-20, and 2 g/liter of BSA (ELI-buffer). The immunoplates were subsequently incubated with an antibody dilution (3 h, 100 μl per well), sheep anti-mouse antibodies labeled with biotin (Sigma, USA; 0.7 μg/ml in ELI-buffer, 100 μl per well, 1 h), and finally with avidin coupled to alkaline phosphatase (Sigma; 0.5 μg/ml in ELI-buffer, 100 μl per well, 1 h) at 37°C. Color reaction was performed using sodium *p*-nitrophenylphosphate hexahydrate (1 mg/ml), dissolved in 97 g/liter of diethanolamine buffer containing 100 mg/liter of $MgCl_2 \cdot 6H_2O$, pH 9.8. The reaction was terminated after 30 min incubation at room temperature by adding 2 M NaOH (50 μl per well). The absorption was registered using a plate reader at 405 nm. Additionally, the Babs activity was measured using bispecific enzyme-linked immunosorbent assay (ELISA) as described earlier [24], which verified the ability of Babs to simultaneously bind two antigens.

5. Analysis of antibody binding to immobilized antigen using an optical biosensor.

5.1. Optical biosensor IAsys. The detection of binding parameters between the antibody and immobilized antigen was performed in this work using a resonant mirror biosensor IAsys produced by Fisons Applied Sensor Technology, UK (FAST). The modern biosensors allow monitoring the antigen–antibody interaction at the phase boundary in real time and without use of labeled reagents [14-17]. The principles of IAsys functioning are as follows: the biosensor surface is irradiated by a laser and the fast dissipating field (evanescent wave) is generated within the surface layer, which allows detection of minor changes of the refractive index and/or thickness of the biosensing surface layer (resonant mirror). For example, during the immunological antibody reaction with immobilized antigen, the thickness of surface layer increases and therefore increases the response registered by the biosensor. The physical principals and construction of the device were described in detail earlier [16]. The collected experimental data were processed and stored using the software supplied with the device (IAsys software, FAST). All the measurements were performed at 25°C. The construction of the IAsys biosensor allows a single moveable cuvette to be placed inside the sensing chamber of the device. The working volume in the cuvette was 200 μl for all experiments. The IAsys biosensor uses an efficient vibro-stirring, with a speed of cuvette 7200 rpm (60 units of the device).

5.2. Immobilization of antigens (HRP and hIgG1) on the surface of IAsys biosensor cuvette. HRP and hIgG1 were covalently coupled to the surface of the analytical cuvette (FAST) and covered with carboxymethyl dextran using the technique described earlier [18, 25]. The coupling chemistry is based on the attachment of antigen amino group to the carboxyl group of dextran. After the activation of carboxymethyl dextran by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (FAST) and N-hydroxysuccinimide (FAST), HRP or hIgG1 at the final concentration of 25 μg/ml in 0.01 M acetate buffer, pH 5.5, were added to the reaction mixture. Unreacted dextran carboxyl groups were blocked with 1 M ethanolamine, pH 8.5 (FAST). Before further use, noncovalently bound protein was removed from the cuvette by rinsing with 0.05 M HCl. Immobilization curves were processed and stored using IAsys software (data not shown). If the absolute value of the response given by immobilized antigen is known, the surface antigen concentration can be calculated using the IAsys software manual supplied with the biosensor, as described by Davis [16]. The HRP response was 1700 arc second, corresponding to HRP surface concentration of 10.5 ng/mm^2 or $(26 \pm 2) \cdot 10^{-14}$ mol/mm^2. The hIgG response was 5500 arc second, corresponding to hIgG1 surface concentration of 33.5 ng/mm^2 or $(21 \pm 1) \cdot 10^{-14}$ mol/mm^2.

5.3. Kinetics of antibody binding to immobilized HRP and hIgG1. Antibody binding to immobilized HRP and hIgG1 was registered after the addition of a large excess of antibodies compared to immobilized antigen (antibody concentration range was 3-1031 nM). The analytical cuvette with immobilized antigen was equilibrated by 180-195 μl of 0.025 M sodium-phosphate buffer, pH 7.4, with 0.15 M NaCl and 0.5 ml/liter of Tween-20 (Na-PBS/T) for at least 5 min. After that, 5-20 μl of antibody solution was added to the cuvette and binding was monitored for 5-10 min. The dissociation was registered for 5-15 min after the removal of antibody solution and single-time addition of 200 μl of Na-PBS/T. The cuvette was regenerated by 0.05 M HCl for 2 min. All antibody samples were analyzed at five to eight different concentrations. The collected experimental data were analyzed using the software supplied with the device (IAsys software).

The data analysis was performed by the FASTfit program, which is specifically designed for mathematical processing of association and dissociation curves obtained from an IAsys biosensor. The program allows the user to select the areas of the graphs (analysis regions) covering the baseline, association, and dissociation. The user can also select the starting points of association and dissociation. In all experiments the base line was analyzed for at least 2 min prior to the association. The first point of association analysis region was 5 sec after the addition of antibody solution. During this time the solution was mixed and the response shift caused by buffer change after the antibody addition was finished. The association was analyzed for 300 sec after the addition of antibodies. The dissociation analysis region was started 10 sec after the removal of solution containing unbound antibodies and addition of the buffer. Dissociation was analyzed for 300 sec after the addition of the buffer.

6. Theoretical basis of the analysis of antibody binding to immobilized antigen.

6.1. Equilibrium binding analysis of antibodies and solid-phase antigen. The IgG antibody molecule is bivalent and capable of simultaneous binding with two solid-

phase antigens under certain conditions. However, a simplified model is usually applied for quantitative description of the interactions between Mabs and immobilized antigen. In this model antigen–antibody interaction is assumed as a reversible, homogeneous (since going on in the solution), single-step process, exhibiting equal binding valence. Under these considerations:

$$K_{ass} = \frac{[B]}{([Ab]_0 - [B])([Ag]_0 - [B])}, \quad (1)$$

where K_{ass} is the equilibrium association constant, assuming homogeneous (since going on in the solution), single-step process characterized by homogeneous antibody valence, M^{-1}; $[Ab]_0$ is total antibody concentration, M; [B] is concentration of bound antibodies, M; $[Ag]_0$ is total concentration of antigen, M.

6.2. Kinetic analysis of antibody binding with solid-phase immobilized antigen. The simplified model is also used for the determination of antibody association (k_{ass}) and dissociation (k_{diss}) rate constants with immobilized antigen, assuming single-step binding with homogeneous valence. In this case the rate of antigen–antibody complex formation is expressed by the following equation:

$$\frac{d[B]}{dt} = k_{ass}[Ab][Ag] - k_{diss}[B], \quad (2)$$

where [Ag] is the concentration of free antigen ($[Ag] = [Ag]_0 - [B]$); [Ab] is the concentration of free antibodies ($[Ab] = [Ab]_0 - [B]$); k_{ass} is the observed association rate constant, $M^{-1}\cdot sec^{-1}$; k_{diss} is the observed dissociation rate constant, sec^{-1}; t is time, sec. It is also assumed that antibodies are in excess toward the antigen ($[Ab]_0 >> [Ag]_0$), and the concentration of antibodies is not varied with time ($[Ab] \approx [Ab]_0 = const$). Antigen–antibody interaction takes place under the pseudo-first order reaction conditions [18], and therefore the solution of Eq. (2) is given by:

$$[B] = [B]_\infty (1 - e^{-k_{on}t}), \quad (3)$$

where $[B]_\infty$ is the equilibrium concentration of antigen–antibody complex at given antibody concentration ([Ab]), which is:

$$[B]_\infty = \frac{[Ab][Ag]_0}{[Ab] + K_{diss}}, \quad (4)$$

where K_{diss} is the observed equilibrium dissociation constant, M; k_{on} in Eq. (3) is the observed rate constant, sec^{-1}; which is:

$$k_{on} = k_{ass}[Ab] + k_{diss}. \quad (5)$$

Certain values of k_{on} and $[B]_\infty$ correspond to each antibody concentration ([Ab]). Calculated from the linear dependence in Eq. (5) (k_{on} on [Ab]), k_{ass} equals to the slope of the straight-line fit. Theoretically k_{diss} can also be determined from Eq. (5) as the interval on the y-axis between the origin and intersection point of the straight line fit in Eq. (5) with the y-axis. In practice, the error of calculation of the constant according to this method of is very high. However, if the dissociation is considered being irreversible (taking place in a sufficiently large volume) and there is no rebinding of free antibodies, then the dissociation can be described by following equation:

$$\frac{d[B]}{dt} = -k_{diss}[B]. \quad (6)$$

The solution of this equation is:

$$[B] = [B]_0 e^{-k_{diss}t}, \quad (7)$$

where $[B]_0$ is the concentration of antigen–antibody complex at the start time.

The observed association (k_{ass}) and dissociation (k_{diss}) rate constants are related to the observed equilibrium association constant (K_{ass}, M^{-1}) and observed equilibrium dissociation constant (K_{diss}, M) by the following equation:

$$K_{ass} = \frac{1}{K_{diss}} = \frac{k_{ass}}{k_{diss}}. \quad (8)$$

6.3. Transformation of the kinetic curves obtained using the optical biosensor. Concentration of the antigen–antibody complex [B] is measured by the IAsys biosensor directly as the response (R) in arc seconds. Equation (3) describing the association of antibodies with immobilized antigen according to a pseudo-first order reaction is transformed in the FASTfit program (section 5.3 in "Materials and Methods") as following:

$$R = R_0 + E(1 - e^{-k_{on}t}), \quad (9)$$

where R_0 is the signal at $t = 0$ (arc seconds). This parameter is intentionally introduced in order to correct possible initial shift of the response caused by buffer change after the addition of antibody solution. E is called the reaction degree and is analogous to $[B]_\infty$ in Eqs. (3) and (4) (arc seconds).

Equation (7) describing irreversible antibody dissociation from the immobilized antigen is transformed by the FASTfit program into:

$$R = R_0^{diss} e^{-k_{diss}t}, \quad (10)$$

where R_0^{diss} is signal at $t = 0$ (arc seconds), this value is analogous to $[B]_0$ in Eq. (7). The FASTfit program applies the repeating processing of experimental association and dissociation data to obtain the corresponding values in Eqs. (9) and (10). The error estimation in this work was performed by matrix inversion [26].

6.4. Model of Mabs bivalent binding with solid-phase immobilized antigen. In reality, a mixture of bivalently and monovalently bound Mabs molecules may exist on the solid phase surface. In this case, solid-phase techniques evaluate Mabs avidity under given experimental conditions, instead of the true avidity of a particular antigen-binding site of the antibody toward the antigen epitope [2, 10-13]. Schematics of antibody–antigen interaction are given in Fig. 1.

The monovalent binding process is characterized by the equilibrium constant (K_1):

$$K_1 = \frac{k_1}{k_{-1}} = \frac{[AbAg]_s}{2[Ab][Ag]_s}, \ M^{-1}, \qquad (11)$$

where k_1 is the association rate constant between antigen-binding site of antibody and antigen, $M^{-1}\cdot sec^{-1}$; k_{-1} is the dissociation rate constant for monovalent complex, sec^{-1}; s is the index indicating the surface concentration, and the absence of this index implies the bulk concentration. The conversion of the bivalent complex into the monovalent is characterized by the equilibrium constant (K_2):

$$K_2 = \frac{k_2}{k_{-2}} = \frac{2[AbAg_2]_s}{[Ag]_s[AbAg]_s}, \ cm^2/mol, \qquad (12)$$

where k_2 is the association rate constant of conversion from monovalent complex into the bivalent, $cm^2\cdot mol^{-1}\cdot sec^{-1}$; k_{-2} is the dissociation rate constant between bivalently and monovalently bound antibodies, sec^{-1}.

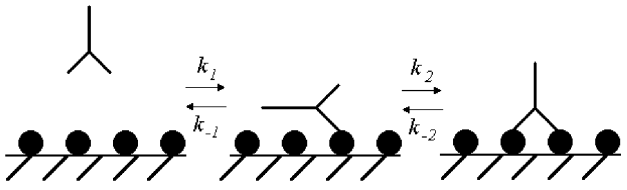

Fig. 1. Schematics of bivalent antibody binding. Antibodies in solution [Ab] bind reversibly to the vacant antigens on the surface [Ag_s] ("s" subscript indicates the surface concentration, the absence of subscript indicates the bulk concentration) to form a monovalently bound antibody–antigen complex $[AbAg]_s$. If there are any vacant antigens on the surface close to the monovalently bound complex, the second antibody binding site may interact reversibly with the second antigen to form a bivalently bound complex $[AbAg_2]_s$ [2].

The statistical factor of two is due to the fact that at the first stage IgG can bind with antigen by either of its shoulders, but it can dissociate by only one; at the second stage the inverse statement is true. The second stage of the reaction presented in Fig. 1 is impossible for Babs and, since they have only one binding site toward the antigen, the statistical factor is one.

Now let us combine the model of bivalent binding (Eqs. (11) and (12)) with the model from Eq. (1), there antigen–antibody interaction is assumed to be reversible, homogeneous, single-step process with homogeneous binding valence. The expression for the observed equilibrium association constant (K_{ass}) is given by:

$$K_{ass} = \frac{k_{ass}}{k_{diss}} = \frac{[AbAg_2]_s + [AbAg]_s}{[Ab][Ag]_s} =$$

$$= K_1 (2 + K_2 [Ag]_s), \ M^{-1}. \qquad (13)$$

Hence, as Eq. (13) shows, the observed parameters of parental Mabs binding with solid-phase antigen are not true physical values and depend on the experimental conditions. The value of true equilibrium association constant (K_1) in solid-phase techniques can be measured using monovalent Babs (or their analogs, Fab-fragments). If the bivalent binding of Mabs is not possible for some reason, and Mabs are only binding monovalently (i.e., in Eq. (13) $K_2 = 0$), then K_{ass} for parental Mabs would be equal to $2K_1$, i.e., K_{ass} for parental antibodies ($2K_1$) would be 2 times higher than K_{ass} for Babs (K_1). If bivalent Mabs binding takes place, the value of K_{ass}, as it derives from Eq. (13), will be significantly higher than $2K_1$ ($K_{ass} >> 2K_1$) and K_{ass} of parental antibodies will much more than 2 times exceed K_{ass} for Babs (K_1).

Based on the comparison of kinetic parameters obtained for bivalent Mabs and monovalent Babs it is possible (as illustrated further on) to determine whether parental Mabs are able to bind bivalently with immobilized antigen and, if that is the case, to estimate the amount of bivalently and monovalently bound Mabs molecules.

6.5. Calculation of the ratio for monovalently and bivalently bound antibody amount. Given that bivalent binding of parental Mabs with immobilized antigen and using Eq. (12), it is possible to calculate the ratio between monovalent and bivalent bound antibodies:

$$\frac{[AbAg_2]_s}{[AbAg]_s} = \frac{K_2[Ag]_s}{2}. \qquad (14)$$

The value of $K_2[Ag]_s$ is often referred to in the literature as the "enhancement factor" [2, 11]. As seen from Eq. (14), this value is equal to the double ratio between monovalently and bivalently bound antibodies. The ratio

between monovalently and bivalently bound antibodies can be calculated using Eq. (13) provided that the K_{ass} values for Babs, equal to K_1, and the K_{ass} value for parental Mabs are known.

6.6. Prediction of theoretically expected variations of the observed kinetic constants of antibody binding with immobilized antigen. The equations presented earlier also allow comparing the kinetic parameters for parental Mabs and Babs binding. The observed association rate constant (k_{ass}) for Babs is equal to k_1 from Eq. (11). The k_{ass} value for parental Mabs in most cases is not dependent on the presence or absence of bivalent binding [11, 13], and is equal to $2k_1$. It should be emphasized that regardless of the presence or absence of bivalent binding, the association rate constant for parental Mabs ($2k_1$) is 2 times higher than k_{ass} for Babs (k_1).

The observed dissociation rate constant (k_{diss}) for Babs is k_{-1}. The k_{diss} value for parental antibodies is obtained from Eq. (13):

$$k_{diss} = \frac{k_{-1}}{K_2[Ag]_s/2 + 1}. \quad (15)$$

Hence, the dissociation of parental Mabs, able to bivalently bind with immobilized antigen, is remarkably lower than Babs dissociation. If the bivalent binding of Mabs with immobilized antigen is not possible for some reason, the dissociation rates for parental Mabs and Babs will be equal.

RESULTS

Kinetic parameters for Babs and parental Mabs binding to immobilized hIgG1 and HRP were compared using an IAsys optical biosensor [14-16]. Figures 2a and 2b present the antibody binding curves with immobilized HRP and human IgG1, obtained by the optical biosensor. The analytical cuvette with immobilized antigen was equilibrated with buffer, then antibodies were added and association was allowed to proceed for 5 min. Antibody dissociation process was monitored after the removal of antibody solution and single-time addition of buffer into the cuvette (see the details in section 5.3 of "Materials and Methods"). The biosensor allows determining the rate constants (k_{ass} and k_{diss}), and also the observed equilibrium association constant (K_{ass}) of parental Mabs and Babs with immobilized antigen based on Eqs. (8)-(10) (sections 2 and 3).

The FASTfit program processes each experimental association curve applying Eq. (9) (section 3). To achieve the best experimental association curve fit by Eq. (9), either relatively low antibody concentrations, not exceeding the value of $10K_{diss}$, or the concentration giving the minimal registered signal should be used. Figure 3 illustrates that with the correctly chosen antibody concentration, an experimental association curve matches with a theoretical curve from Eq. (9). The top graph shows the response dependence on time for bispecific monoclonal antibody (anti-HRP/hIgG) binding with immobilized hIgG1, at

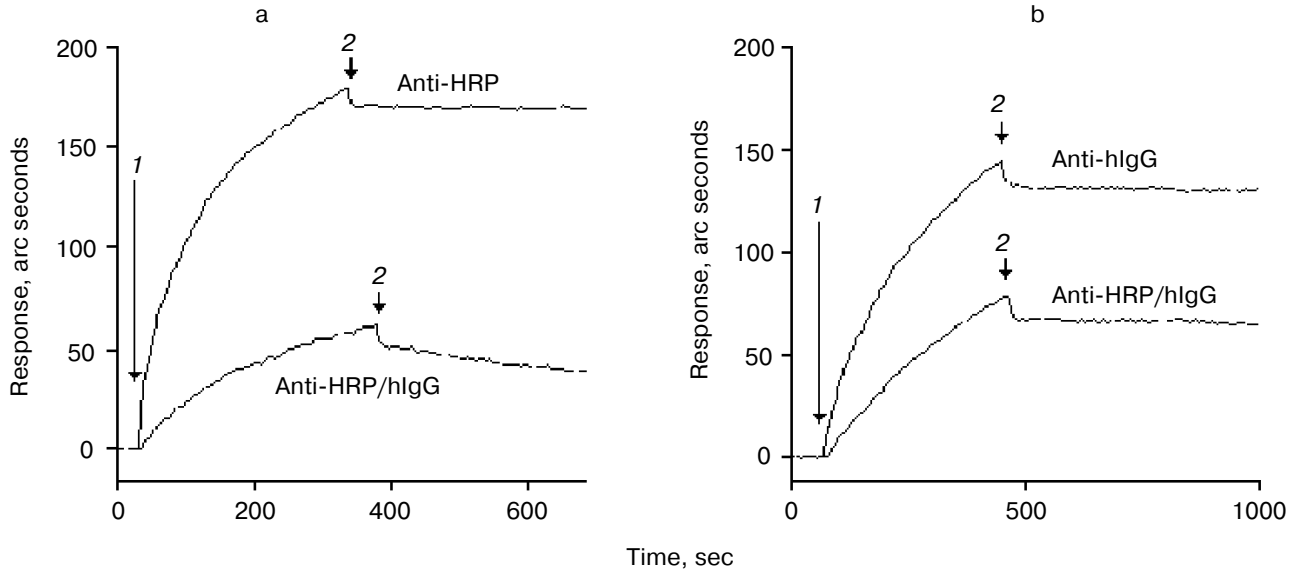

Fig. 2. Parental monoclonal and bispecific monoclonal antibodies binding curves with immobilized HRP and human hIgG1 obtained using an optical biosensor. The analytical cuvette with immobilized antigen was equilibrated with the buffer. After that the antibodies were added to the cuvette (*1*) and allowed to bind for 5 min. The dissociation of antibodies was registered after the removal of antibody solution and single-time addition of the buffer (*2*) for 5 min. See section 5.3 of "Materials and Methods" for details; a) association and dissociation of bispecific monoclonal antibodies (clone 36F9/75G5, antibody concentration 490 nM) and parental monoclonal antibodies (clone 36F9, antibody concentration 490 nM) with immobilized HRP; b) association and dissociation of parental monoclonal antibodies (clone 75G5, antibody concentration 34 nM) and bispecific monoclonal antibodies (clone 36F9/75G5, antibody concentration 34 nM) with immobilized human IgG1.

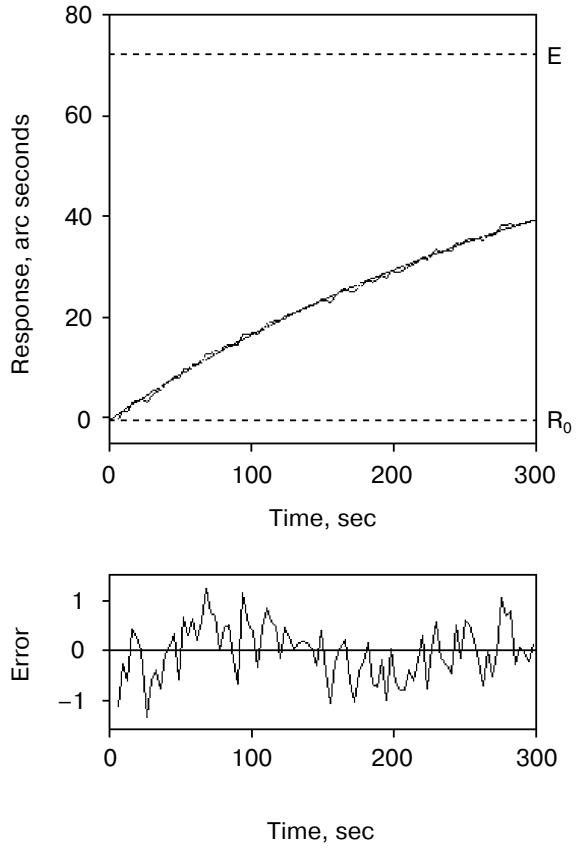

Fig 3. Analysis of antibody binding to the immobilized antigen. Antibody concentration is close to the K_{diss} value. Experimental curves were processed using Eq. (9) (section 3) and the FASTfit program. The top graph shows the comparison of the experimental data from association of 7 nM parental antibodies with immobilized hIgG1 (broken line) to Eq. (9) curve (smooth line). The curves match ($\chi^2 = 0.24$). The calculated value for k_{on} is $(1.98 \pm 0.09)\cdot 10^{-3}$, that for E is 73 ± 2, and for R_0 is -0.80 ± 0.18. The bottom graph illustrates the error of the curve fitting against time for the given experimental curve, showing that deviations of experimental data from the values calculated using Eq. (9) do not exceed 1.5 arc second.

antibody concentration comparable to K_{diss} value. As can be seen, the experimental data are well fit by the theoretical curve from Eq. (9), and the bottom graph illustrating the error dependence on time shows negligible deviation.

However, if antibody concentration significantly exceeds the value of $10K_{diss}$, the experimental curves can no longer be described by Eq. (9). Under these conditions R_0-value and error value χ^2 would substantially increase, meanwhile k_{on} has an underestimated value (data not shown). Association curves are no longer described by Eq. (9) also at antibody concentrations lower than $10K_{diss}$, when association time is longer than 10 min (data not presented). Similar phenomena were also reported in other publications [18, 26, 27]. The deviation of experimental curves from Eq. (9) at high antibody concentrations or at long analysis time is explained by either saturation of immobilized antigen with antibodies [18, 27] or by diffusion limitations. In the present study the diffusion effects were not the limiting factor since the propeller speed in all experiments was 7200 rpm, which is close to the maximally possible mixing speed for this instrument (12,000 rpm). A number of particular experiments proved that an increase in mixing speed up to 10,800 rpm did not lead to a noticeable change of R_0, E, and k_{on} parameters of the experimental association curve (data not presented).

Experimental association curves were obtained for at least five different antibody concentrations and k_{on} value was calculated from each of the curves. The k_{ass} value was found for each antibody concentration from the linear k_{on} dependences on $[Ab]_0$ (Eq. (5), section 2). Typical plots are presented in Figs. 4a and 4b. When antibody concentrations significantly exceed the value of $10K_{diss}$, the k_{on} obtained from experimental association curves has an underestimated value. It becomes noticeable when k_{ass} is calculated according to Eq. (5), because k_{on} obtained at high antibody concentration were found to be lower than predicted by other experimental data points. These k_{on} values were omitted and the final k_{ass} value was calculated using remaining data (table, Figs. 4a and 4b).

Parameters of antibody binding with immobilized antigens (M ± SD)

Antibody specificity	Immobilized antigen	Biosensor, IAsys			RIA
		k_{ass}, $M^{-1}\cdot sec^{-1}$	k_{diss}, sec^{-1}	$K_{ass} = k_{ass}/k_{diss}$, M^{-1}	K_{ass}, M^{-1}
Anti-HRP	HRP	$(1.9 \pm 0.1) \cdot 10^4$	$(4.0 \pm 0.1) \cdot 10^{-5}$	$(4.8 \pm 0.3) \cdot 10^8$	$(2.0 \pm 0.1) \cdot 10^8$
Anti-hIgG/HRP	HRP	$(8.0 \pm 1.0) \cdot 10^3$	$(8.3 \pm 0.1) \cdot 10^{-4}$	$(9.6 \pm 1.2) \cdot 10^6$	$(5.3 \pm 0.5) \cdot 10^6$
Anti-hIgG	hIgG1	$(1.5 \pm 0.2) \cdot 10^5$	$(2.7 \pm 0.1) \cdot 10^{-4}$	$(5.5 \pm 0.7) \cdot 10^8$	$(5.9 \pm 0.6) \cdot 10^8$
Anti-hIgG/HRP	hIgG1	$(6.8 \pm 0.5) \cdot 10^4$	$(3.0 \pm 0.1) \cdot 10^{-4}$	$(2.3 \pm 0.2) \cdot 10^8$	$(2.6 \pm 0.3) \cdot 10^8$

Note: The rightmost column contains the equilibrium association constants (K_{ass}) calculated using radioimmunological assay (RIA). See our previous work [1] for details.

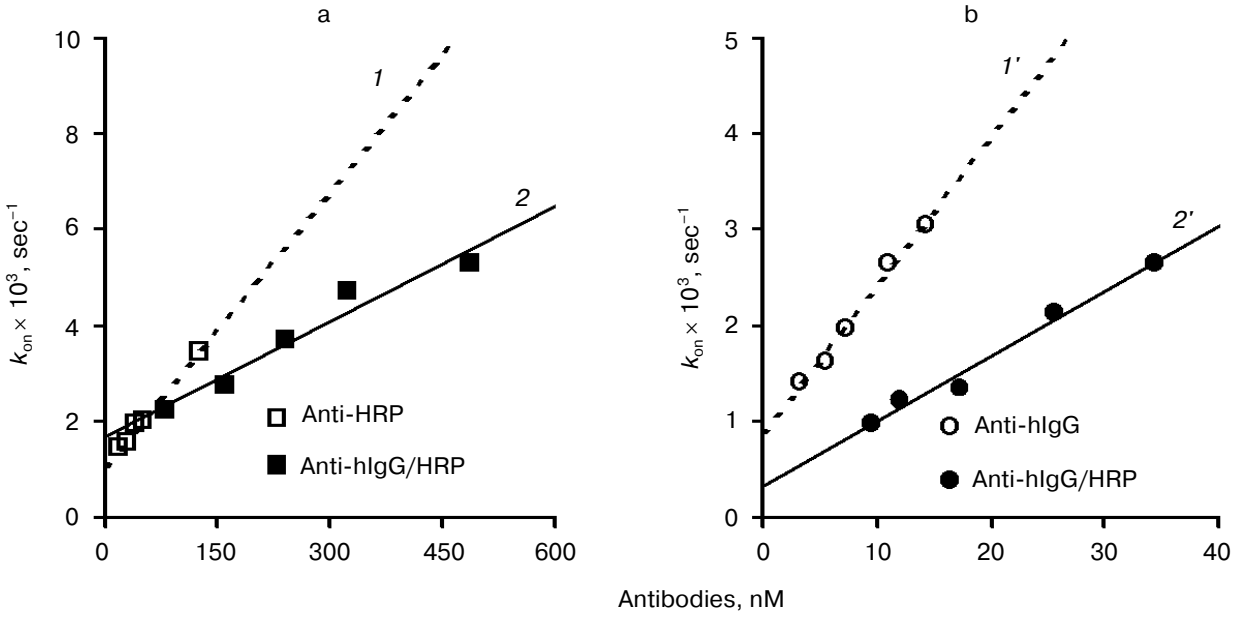

Fig. 4. Dependence of k_{on} on antibody concentration obtained using the biosensor. The slopes of the straight lines are equal to the observed association rate constant (k_{ass}) from Eq. (5) (section 2). The k_{on} for each concentration of antibodies was calculated using Eq. (9) (section 3). a) k_{on} dependence on parental anti-HRP antibody (clone 36F9) and bispecific monoclonal antibody (clone 36F9/75G5) concentration for binding with immobilized HRP; *1*) anti-HRP antibodies (clone 36F9); *2*) anti-HRP/hIgG antibodies (clone 36F9/75G5); b) k_{on} dependence on parental antibodies against hIgG (clone 75G5) and bispecific monoclonal antibodies (clone 36F9/75G5) for binding with immobilized hIgG1; *1′*) anti-HRP antibodies (clone 36F9); *2′*) anti-HRP/hIgG antibodies (clone 36F9/75G5).

The experimental dissociation curves (Fig. 2, a and b) were analyzed using Eq. (10) (section 3). Antibody dissociation was registered after removal of antibody solution from the cuvette (after antibody association) and single-time addition of the buffer (section 5.3 in "Materials and Methods"). A certain amount of antibodies left in the dead volume of the cuvette may also interact with an antigen and distort the association parameters. To avoid errors caused by possible binding of remaining antibodies, the value of k_{diss} was calculated using only experimental dissociation curves registered as a result of antibody binding at concentrations close to K_{diss}. In this case, the binding of antibodies remaining in the dead volume is not registered by the biosensor because the concentration of antibodies in the solution is negligible (<0.03 K_{diss}).

Figures 2a and 2b present the experimental association and dissociation curves of parental Mabs and Babs with immobilized antigens obtained by the optical biosensor. The k_{ass} value for anti-HRP Mabs is 2.4 higher than k_{ass} for anti-HRP site of Babs (table, Fig. 2a), whereas k_{ass} value for parental Mabs against hIgG is 2.2 higher than k_{ass} for anti-hIgG site in Babs (table, Fig. 2b). Theoretically, k_{ass} for parental Mabs has to be 2 times higher than k_{ass} for Babs (section 6). The difference between theoretical and experimental values of k_{ass} do not exceed the calculation error (see the table). At the same time, k_{diss} for anti-HRP site of Babs is 21 times higher than k_{diss} for anti-HRP Mabs (table, Fig. 2a), which indicates that anti-HRP Mabs are bound bivalently to the immobilized HRP (section 6). The K_{ass} for anti-HRP Mabs exceeds 50 times K_{ass} for anti-HRP site of Babs. If constant ratio for Mabs and Babs is known, the ratio of bivalently and monovalently bound antibodies for parental Mabs can be calculated using Eqs. (13) and (14) (sections 4 and 5). This ratio is equal to 24. Hence, at given antigen density, 96% of the total Mabs amount on the biosensor surface are bound bivalently, whereas only 4% are bound monovalently. On the contrary, the dissociation rates for parental Mabs against hIgG and anti-hIgG site of Babs are practically identical (table, Fig. 2b), which shows that no bivalent binding of parental Mabs with immobilized hIgG1 occurs (section 6). The K_{ass} for parental Mabs against hIgG determined using the biosensor is 2.4 times higher than K_{ass} for anti-hIgG site of Babs, which is close to the theoretically expected value (section 4).

DISCUSSION

Bivalent binding was investigated in detail by a comparison of binding of native antibodies and their Fab-fragments to immobilized antigens [2, 10-13], also using

optical biosensors [16-19, 27, 28]. It should be emphasized that the comparison of Fab-fragments and whole IgG molecules binding is not sufficiently valid model for the investigation of bivalent binding. The disadvantage of this comparison implies that the structure of Fab-fragments is different from that of native antibody molecules, since it is just a "half" of immunoglobulin molecule lacking the Fc-fragment. However, there are data indicating that the Fc-region may have importance for antigen-binding ability of antibodies (e.g., it may affect flexibility of the molecule) [29]. In our opinion, the ideal model for investigation of binding between antibodies and immobilized antigens is the comparative binding analysis of Babs and parental Mabs. The Babs molecule structure is identical to that of the native immunoglobulin molecule (at least when both "halves" of Babs belong to the same IgG sub-class) [6]. Mabs analyzed in this study belong to IgG1 sub-class (section 1 of "Materials and Methods"); also, both "halves" of Babs (anti-hIgG and anti-HRP) belong to IgG1 sub-class. It can be assumed that in the case of parental Mabs and Babs binding analysis was based on molecules with the same native structure. Babs molecules are capable of only monovalent binding with a solid-phase immobilized antigen; meanwhile the parental Mabs are capable of both bivalent and monovalent binding. The formation of Babs with altered affinity of antigen-binding sites due to the "incorrect" association of H- and L-chains is a very rare [30] but yet possible event [31]. On the other hand, the true affinity of antibody–antigen binding can be easily measured in solution. However, the solid-phase immobilization of antigen (e.g., on the surface of an immunoplate) leads to a partial distortion of protein conformation, changing its binding properties [32]. Therefore, an absolute value of affinity constant measured in solution is not true for solid-phase experiments.

Based on the proposed model for bivalent binding (section 4), it is possible to conclude that anti-HRP Mabs are bound bivalently to HRP. At the same time, the bivalent binding of Mabs against hIgG with adsorbed hIgG1 was not observed. The absence of bivalent binding with hIgG1 can be explained by relatively large size of the hIgG molecule (the M_r of hIgG is 4 times higher than the M_r of HRP). Furthermore, the HRP molecule has a globular structure, while the hIgG molecule has a "stick" structure, which may also affect the binding parameters. The ability of Mabs to bivalently bind with immobilized antigen is mainly dependent on the steric interactions between the antigen-binding site of Mabs and the corresponding antigenic epitope. For instance, it was demonstrated [11] that different Mabs specific to the same antigen may display different binding patterns, either bivalent or monovalent. Thus, the presence or absence of bivalent antibody binding to immobilized antigen should be proved not only for each antigen, but also for each Mabs producing clone toward the given antigen.

It should be observed that equilibrium association constants of antibodies (K_{ass}) determined with the use of the biosensor correlate well with the constants measured in our previous work [1] by radioimmunological technique (table). This argues for their correctness, despite the fact of different antigen immobilization techniques (covalent attachment to the biosensor surface or physical adsorption on the polystyrene surface for radioimmunological assay).

Also noticeable is the extremely low dissociation rate for anti-HRP Mabs. The half-life time for antigen–antibody complex ($t_{1/2}$), which is given by:

$$t_{1/2} = \frac{\ln 2}{k_{diss}}, \text{ sec}, \qquad (16)$$

is approximately 5 h for anti-HRP Mabs. From the practical point of view, it is acceptable to assume that the bivalent binding is almost irreversible. It is not possible to detect any significant dissociation of anti-HRP Mabs during the time comparable to the time of immunological analysis (1-2 h). The same phenomenon was also observed in another work [33], where it was demonstrated that $t_{1/2}$ is more than a week for antibodies bound to polyvalent antigen on the plastic surface.

Babs were considered as effective markers for solid-phase analysis techniques in a number of investigations [3-5]. One of the first described were antibodies with specificity to anti-somatostatin/anti-HRP [31]. It was claimed that they are as efficient in immunohistochemical tests for somatostatin producing cells as parental Mabs toward somatostatin. Sandwich methods of antigen testing were described later, where Babs with specificity toward the tested antigen and enzyme label was used instead of traditional enzyme conjugate (a detailed summary of the relevant publications is presented in a recent review [5]). The authors of the mentioned publications also claimed that Babs result in a significantly improved sensitivity in a comparison with the labeled parental Mabs. The data presented in this work conveniently illustrate that the Babs cannot provide a gain in sensitivity compared to the parental Mabs in all non-competitive assays based on antibody binding with a solid-phase antigen, since Babs are not able to bind bivalently. In another recent work, the efficiency of traditional conjugate was compared to the efficiency of Babs in sandwich method for myoglobin detection [34]. The drastic decrease in both the assay sensitivity and antigen detection limit was demonstrated for Babs in a comparison with traditional conjugate in enzyme-linked immune sandwich.

In our opinion, the use of Babs as labeled antibodies in solid-phase systems for antigen analysis is inadvisable. The obtaining of antibody producing clones is a time-consuming procedure that requires skillful personnel.

Certain problems may also emerge during the purification of Babs. Meanwhile, improvement of analytical system parameters can hardly take place.

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