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The comparison of the ability of monoclonal antibodies directed to different proteins (human IgG, human myoglobin and HRP) and bispecific antibodies derived thereof to bind antigens immobilized on a surface of a solid phase

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Abstract

Background: Bindings of mouse monoclonal antibodies (mAbs) and affinity purified bispecific antibodies (bAbs), derived thereof, to antigens adsorbed on immunoplates have been compared, using ELISA and RIA methods. *Methods*: The analysed panel of antibodies included mAbs specific to human myoglobin (Mb), human IgG (hIgG) and horseradish peroxidase (HRP) and biologically produced bAbs with double specificity to Mb and HRP, and to hIgG and HRP. *Results*: The degree of difference between different mAbs and corresponding bAbs varied markedly from antibody to antibody, depending on whether the parental mAbs could bind immobilized antigens bivalently. The observed equilibrium binding constant (K_{obs}) for anti-HRP mAbs was 21–38 times higher that of anti-HRP site of bAbs (anti-hIgG/HRP or anti-Mb/HRP, respectively), due to bivalent binding of mAbs. Anti-Mb mAbs also bound bivalently with immobilized Mb. On the contrary, anti-hIgG mAbs bound monovalently with immobilized hIgG in the same conditions. The avidity of anti-Mb/HRP bAbs increased, if both antigens were simultaneously adsorbed on a solid phase. *Conclusions*: The obtained data indicate that the use of bAbs in heterogeneous immunoassays instead of traditional mAb-enzyme conjugates hardly can provide the significant gain in assay performance if parental mAbs bind bivalently.

Keywords: ELISA; Immobilized antigen; Bivalent interaction; Affinity; Avidity; Bispecific antibodies

Abbreviations: bAbs, bispecific monoclonal antibodies; hIgG, human IgG; HRP, horseradish peroxidase; K_{obs}, observed equilibrium binding constant, (M^{-1}); mAbs, monoclonal antibodies; Mb, human myoglobin; RIA, radioimmunoassay

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1. Introduction

Bispecific antibodies (bAbs) are immunoglobulin molecules with two different antigen-binding sites. Present methods for obtaining bAbs include chemical, biological and molecular genetic approaches [1,2]. The biological method, considered in this work, makes it possible to prepare lasting cell lines–hybrid hybridomas (tetradomas), which perform the "natural" assembling of bAbs molecules [3]. This is achieved by the fusion of two hybridomas, secreting different monoclonal antibodies (mAbs). Equilibrium binding studies in solutions have shown that bAbs, obtained by cell fusion, usually retain the affinity of parental mAbs [4,5]. BAbs are considered to be the ideal bioconjugates, which can specifically glue any two different molecules together without the need for chemical conjugation [2]. In particular, bAbs bearing both binding sites to the enzyme (i.e., horseradish peroxidase (HRP) or alkaline phosphatase) and the antigen may be used in enzyme immunoassays and immunohistochemistry instead of covalently linked enzyme-mAb conjugates [6]. Another possible important field of applications of bAbs is cell targeting [1,7,8]. The potential advantage of bAbs over conventional mAbs in these fields has been considered in a number of works [6–13]. On the other hand, in many of these applications it is necessary that antibodies should be able to effectively bind immobilized antigens. Two parameters are usually considered to be of special importance for the characterization of the ability of mAbs to bind antigens immobilized on a solid phase: the intrinsic affinity of monovalent binding (true affinity) and the avidity (functional affinity) [14]. The true affinity refers to the interaction between an epitope and the binding site on one arm of a multivalent antibody (bivalent in case of IgG) and can be analysed in conventional mass-action terms in equilibrium binding experiments [14]. Avidity (functional affinity) refers to the interaction of the antibody with the antigen as a whole, and is the final result of a true affinity, antibody valence, antigen density on a solid phase and steric and statistical factors [14,15]. The difference between the intrinsic affinity and avidity is connected with the ability of a multivalent antibody to bind antigens simultaneously with two (in case of IgG) or several antigen-binding sites. It has been demonstrated that the avidity can be significantly augmented due to the bivalent binding of IgG molecule with solid-phase antigen [14,16]. Unlike parental mAbs, biologically produced bAbs are not able to bind bivalently, because they are monovalent molecules having only one binding site for each antigen [3]. However, this possible deficiency of biologically produced bAbs seems to be paid little attention to. Thus, up to now, the studies on the quantitative comparison of the ability of mAbs and bAbs (derived from these mAbs by cell fusion) to bind solid-phase antigens have not been performed. At the same time, the analysis of this question not only seems of importance for application of bAbs, but also may contribute to the illumination of the mechanisms of antigen–antibody interactions on the surface of a solid phase. The bivalent binding has been directly evidenced in a number of works in the course of comparisons of measured affinities for whole molecule antibody and F(ab) fragment of identical origin. F(ab) fragments were used in these experiments as the instrument of measuring the affinity of monovalent binding [16–21]. The use of bAbs, instead of F(ab) fragments, for this purpose seems to be a more advantageous approach, because bAbs, obtained by cell fusion, retain the structure of the intact IgG molecule.

In the present study, we have performed the quantitative analysis of the ability of three various mAbs, and bAbs derived thereof, to bind antigens of different structure: human myoglobin (Mb), human IgG (hIgG) and HRP, immobilized on a surface of a solid phase (immunoplates). The modes of mAbs behaviour in the studied systems have been deciphered using ELISA and solid-phase radioimmunoassay (RIA), with the help of the accepted theoretical binding model [14,16]. Two of three studied mAbs showed a significantly higher functional affinity than bAbs, due to the predominance of bivalent binding, suggesting the advantage of natural antibodies over bAbs in heterogeneous systems.

2. Materials and methods

2.1. Cell lines

Mouse hybridoma and tetradoma cell lines previously obtained in our laboratory were used as the

sources of antibodies: clone 36F9 (anti-HRP mAbs) [22], clone 75G5 (anti-hIgG mAbs) [23], clone 14D6 (anti-Mb mAbs) [24], tetradoma clone 14D6 × 36F9 (anti-Mb/HRP bAbs) [13] and tetradoma clone 75G5 × 36F9 (anti-hIgG/HRP bAbs) [23]. All cell lines produced antibodies of IgG1 subclass, as defined with the Calbiochem hybridoma subisotyping kit.

2.2. Myoglobin purification

Mb was isolated from human heart as described previously [25]. In our study, Mb with 99% purity was used.

2.3. Purification of antibodies

MAbs produced by the 14D6, 36F9 and 75G5 clones were purified by affinity chromatography on antigen-Sepharose from ascites obtained by inoculating mice with the corresponding hybridoma cells. The antigens (Mb, HRP or hIgG) were conjugated to CNBr-activated Sepharose 4B (Pharmacia, Sweden) according to Pharmacia recommendations. The details of the purification procedures were described [5,13,22–24,26]. Anti-Mb/HRP bAbs were isolated from ascitic liquid of the tetradoma 14D6 × 36F9 by successive affinity chromatography on HRP-Sepharose and Mb-Sepharose. Anti-hIgG/HRP bAbs were isolated from ascites of the tetradoma 75G5 × 36F9 by successive chromatography on HRP-Sepharose and hIgG-Sepharose. The purity of antibody preparations was monitored by SDS-PAGE (12.5% acrylamide) according to Laemmli [27]. In the preliminary experiments, we have shown that more than 99% of the affinity-purified antibodies retained their immunological activity, even after repeated affinity chromatography (data not shown).

2.4. Determination of the concentration of proteins

The concentrations of Mb and purified antibodies in solutions were determined by spectrophotometry, assuming that $A^{1\ cm}_{280\ nm} = 17.6$ and $A^{1\ cm}_{220\ nm} = 13.2$ correspond to 10 mg/ml of Mb, and $A^{1\ cm}_{280\ nm} = 14.0$ corresponds to 10 mg/ml of purified antibodies [28]. For the 10 mg/ml solution of purified HRP $A^{1\ cm}_{403\ nm} = 22.75$ and $A^{1\ cm}_{280\ nm} = 7.3$ [29].

2.5. Testing of antigen-binding activity after affinity purification

Anti-hIgG and anti-Mb activity was tested by ELISA, as described previously [23,24]. Anti-HRP activity was measured by the peroxidase–antiperoxidase procedure [22] and using avidin–biotin system, described in Section 2.6. In addition, the activity of bAbs was tested by double-antigen ELISA, as described previously [13,23]. This test confirmed the ability of bAbs to bind two antigens.

2.6. The measurement of the binding of antibodies with immobilized antigens using the immunoenzymatic method

The wells of the removable ELISA 96-well plates (Medpolymer, Russia) were saturated overnight at room temperature with different antigens: HRP ($R_z > 3.0$) (Calbiochem); hIgG1 (donated by T.N. Batalova, Institute of Epidemiology, Moscow); human Mb (for all proteins: concentration 10 μg/ml in 0.05 mol/l sodium carbonate buffer, pH 9.5, 100 μl per well), or with the equimolar mixture of HRP and Mb (3.2 μg/ml of Mb and 6.8 μg/ml of HRP). Dilutions of affinity purified antibodies at the concentrations of 1–4096 ng/ml were prepared. The antibodies were dissolved in 0.025 mol/l sodium phosphate buffer (pH 7.4) with 0.15 mol/l NaCl, 2 g/l BSA and 0.5 ml/l Tween 20 (ELI-buffer). After washing with distilled water (4–5 times), the immunoplates were successively incubated at 37°C with different antibody dilutions (3 h, 100 μl per well, each dilution was tested using four wells), sheep anti-mouse antibodies labeled with biotin (Sigma, USA; 0.7 μg/ml in ELI-buffer, 100 μl per well, 1 h) and avidin, conjugated with alkaline phosphatase (Sigma; 0.5 μg/ml in ELI-buffer, 100 μl per well, 1 h). The colour reaction was developed with disodium salt of *p*-nitrophenyl phosphate hexahydrate (PNPP, Sigma). The PNPP (1 mg/ml) was dissolved in 97 ml/l diethanolamine buffer with 100 mg/l $MgCl_2$, pH 9.8. The incubation (100 μl per well) was carried out for 30 min at room temperature. The reaction was stopped by adding 50 μl of 2 mol/l NaOH. Absorption at 405 nm was measured using a Titertek Multiskan plate spectrophotometer (Flow Laboratories). Two-fold increase of the time of incubation

with antibody dilutions did not result in the increase of the binding (data not shown).

2.7. Iodination of antibodies and their purification

The antibodies were iodinated using the chloramine method (20 MBq ^{125}I per 10 μg of IgG) [30]. Labeled antibodies were affinity purified on the corresponding antigen-Sepharose columns, as described previously [26], with some modifications. The antibodies were eluted with 0.1 mol/l acetic acid, with 2 g/l BSA (the solution was titrated with HCl to pH 2.2). The eluted fraction was neutralized with the concentrated solution of ammonia. ^{125}I-labeled bAbs anti-hIgG/HRP were purified by two-stage affinity chromatography on HRP-Sepharose and hIgG-Sepharose. ^{125}I-labeled bAbs anti-Mb/HRP were purified by successive affinity chromatography on HRP-Sepharose and Mb-Sepharose. Labeled antibodies were stored at 5°C and were used 1 week after preparation.

2.8. Determination of the concentration of ^{125}I-labeled antibodies after affinity purification

The concentration of labeled antibodies was measured as described in Section 2.6. To measure the amount of bAbs anti-hIgG/HRP, their binding with hIgG and HRP was analysed, and the mean concentration was calculated. Similarly, the mean concentration of bAbs anti-Mb/HRP was calculated by the analysis of their binding with Mb and HRP.

2.9. The measurement of binding of ^{125}I-labeled antibodies with antigens adsorbed on a solid phase

The flexible immunoplates (Titertek) were saturated by antigens, as described in Section 2.6 (50 μl of solution per well) and incubated for 3 h at 37°C with ^{125}I-labeled antibodies (1×10^3–1×10^7 cpm/ml), 50 μl of solution per well. Two-fold increase of the time of incubation did not result in the significant increase of the binding (data not shown). The plates were washed with distilled water (4–5 times), and were allowed to dry at room temperature, overnight. Then the wells were cut off, and the radioactivity was measured using a Gamma Trac 1191 counter. To determine the overall amount of ^{125}I-labeled antibodies, the radioactivity was measured in the 50-μl volume samples of each antibody dilution.

2.10. The theoretical basis of the analysis of the binding of antibodies with immobilized antigens

2.10.1. Equilibrium binding analysis of antibody binding with solid-phase antigen

The IgG antibody is bivalent, capable of binding two antigenic sites under favourable conditions. Commonly used forms of solid-phase data analysis model the antibody–antigen interaction as a homogeneous, equilibrium, single-step process and exhibiting homogeneity binding valence [14]. That is:

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

where the terms are: K—equilibrium association constant, assuming single-step, homogenous binding (M^{-1}); $[Ab]_0$—total concentration of antibody (M); [B]—total concentration of bound antibody (M); $[Ag]_0$—total concentration of antigen (M).

Various transformations of Eq. (1) lead to the commonly used forms of antibody binding analysis. One of the most widely used of these methods is the Scatchard plot [31]:

$$\frac{[B]}{[Ab]} = K[Ag]_0 - K[B], \quad (2)$$

where [Ab] is the concentration of free antibody (M), ($[Ab] = [Ab]_0 - [B]$). When the experimental data are plotted in the form of Eq. (2), the slope and x-axis intercept of the best-fit line through the data yield the equilibrium binding constant and the total concentration of antigen. In practice, the experimental binding data are frequently presented in a more simple form—concentration of bound antibody ([B]) vs. total concentration of antibody ($[Ab]_0$). At low [B] (consequently, $[Ag]_0 \gg [B]$), Eq. (1) may be transformed to yield:

$$[B] \approx \frac{K[Ag]_0}{1 + K[Ag]_0}[Ab]_0. \quad (3)$$

These co-ordinates are frequently used in ELISA tests; moreover, the concentration of bound antibody ([B]) is presented not in standard units, but as

absorbance. The tangent of the calibration curve is proportional to the coefficient: $K[\mathrm{Ag}]_0/(1 + K[\mathrm{Ag}]_0)$.

2.10.2. The model of bivalent binding of mAbs with antigens immobilized on the surface of solid phase

In reality, IgG antibodies may exist in a mixture of monovalently and bivalently bound states on the surface of a solid phase. In this case, the solid-phase methods measure the avidity of mAbs in the given experimental conditions rather than the intrinsic affinity of individual antigen-binding site for antigenic epitope. The proposed model of bivalent binding summarizes previous theoretical elaborations on this subject [14–16]. A schematic of the bivalent interaction is presented in Fig. 1.

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

$$K_1 = \frac{k_1}{k_{-1}} = \frac{[\mathrm{AbAg}]_s}{2[\mathrm{Ab}][\mathrm{Ag}]_s}, \quad (\mathrm{M}^{-1}), \tag{4}$$

where the terms are: k_1—association rate constant between antibody and antigen reactive sites (M^{-1} s^{-1}); k_{-1}—dissociation rate constant between antibody and antigen reactive sites (s^{-1}); $[\mathrm{Ag}]_s$—surface concentration of vacant antigenic binding sites; $[\mathrm{AbAg}]_s$—surface concentration of monovalently bound antibody; subscript "s" denotes surface concentration, and a lack of subscript denotes bulk concentration. The conversion of monovalent to bivalent antibody binding is characterized by the equilibrium constant (K_2):

$$K_2 = \frac{k_2}{k_{-2}} = \frac{2[\mathrm{AbAg_2}]_s}{[\mathrm{Ag}]_s[\mathrm{AbAg}]_s}, \quad (\mathrm{cm}^2/\mathrm{mol}), \tag{5}$$

where the terms are: k_2—rate of conversion from monovalent to bivalently bound antibody (cm^2 mol^{-1} s^{-1}); k_{-2}—dissociation rate constant between bivalently and monovalently bound antibody (s^{-1}); $[\mathrm{AbAg_2}]_s$—surface concentration of bivalently bound antibody; for the other terms see notes to Eq. (4).

The statistical factors of two (2) are introduced due to the fact that, in the first step, IgG can bind to either of its arms but dissociate from only one while in the second the converse is true. For bAbs, the second stage is not possible (the statistical factor is equal to one).

The observed equilibrium binding constant (assuming the model of Eq. (1)) for a given set of experimental conditions is given by:

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

$$= K_1(2 + K_2[\mathrm{Ag}]_s), \quad (\mathrm{M}^{-1}), \tag{6}$$

where the terms are: k_{ass}—the observed kinetic association constant (M^{-1} s^{-1}); k_{diss}—the observed kinetic dissociation constant (s^{-1}); for the other terms see Eqs. (2), (4) and (5).

At the presence of bivalent binding, K_{obs} is not a constant physical value, but depends on the experimental conditions (the initial antibody concentra-

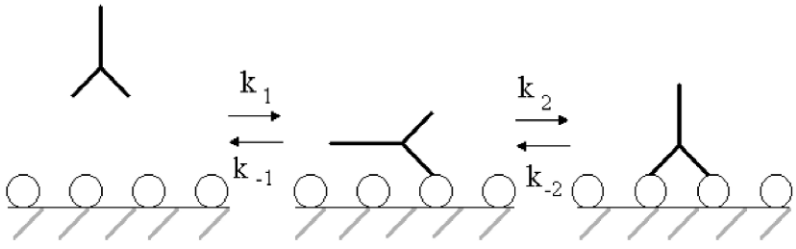

Fig. 1. Schematic of bivalent antibody binding. The bivalent monoclonal antibody in solution [Ab] reversibly binds to a vacant binding site at surface concentration $[\mathrm{Ag}]_s$ (subscript "s" denotes surface concentration, and a lack of subscript denotes bulk concentration) to form a monovalently bound complex. The monovalently bound antibody at surface concentration $[\mathrm{AbAg}]_s$ may then reversibly combine with a vacant antigenic site within arm's reach of the antibody to form a bivalently bound complex at surface concentration $[\mathrm{AbAg_2}]_s$ [14].

tions, surface antigen density). The intrinsic affinity of the monovalent interaction (K_1) in solid-phase experiments may be measured with the help of bAbs (or their analogues—F(ab) fragments). As will be shown in the following sections, bivalent binding may be evidenced by the comparison of the parameters of titration curves and Scatchard plots for bivalent mAbs and bAbs derived thereof.

2.10.3. Use of bivalent model to predict theoretically expected variations of K_{obs}, defined from Scatchard plots

When equilibrium binding data are presented in the form of Scatchard plots, K_{obs} may be defined from the value of the tangent of the slope of the best-fit line through the data (Eq. (2)). If, for some reasons, only monovalent binding occurs ($K_2 = 0$, Eq. (6)), then K_{obs} for parental mAbs will be equal to $2K_1$. As bAbs molecule carries only one binding site for each antigen, K_{obs} for bAbs will be equal to K_1. If a bivalent binding is taking place, then K_{obs}, as follows from Eq. (6), will be higher than $2K_1$ ($K_{obs} \gg 2K_1$).

2.10.4. Use of bivalent model to predict theoretically expected variations of calibration curves parameters, depending on the absence or presence of bivalent binding of antibodies with immobilized antigens

When the experimental data are plotted in the form of Eq. (3) (concentration of bound antibody vs. total antibody concentration, [B] vs. $[Ab]_0$), the tangent of the slope of the binding curve to abscissa constitutes $K_{obs}[Ag]_0/(1 + K_{obs}[Ag]_0)$. In the absence of bivalent binding, the value of K_{obs} for the parental mAbs will be equal to $2K_1$, which is two times higher K_{obs} for bAbs, equal to K_1. Consequently, binding curve coefficient for parental mAbs will constitute $2K_1[Ag]_0/(1 + 2K_1[Ag]_0)$, and coefficient for binding curve of bAbs will be equal to $K_1[Ag]_0/(1 + K_1[Ag]_0)$. The value $[Ag]_0$ is the same for bAbs and mAbs. Therefore, in the absence of bivalent binding the ratio of binding curve coefficient for mAbs and bAbs will constitute $(2 + 2K_1[Ag]_0)/(1 + 2K_1[Ag]_0)$. Upon further algebraic rearrangements, the following expression is achieved for this ratio: $1 + 1/(1 + 2K_1[Ag]_0)$. As $2K_{obs}[Ag]_0 > 0$, in the absence of bivalent binding the ratio of binding curves coefficients for parental mAbs and bAbs cannot exceed 2. If this ratio overdraws the level of 2, this may give evidence for the deviation from homogeneous valence due to bivalent binding of the certain portion of mAb molecules.

2.10.5. The ratio of bivalently and monovalently bound antibodies

The described model of bivalent binding may be applied in order to determine the percentage of bivalently and monovalently bound parental mAbs on the surface of a solid phase. From Eq. (5), this ratio may be given by:

$$\frac{[AbAg_2]_s}{[AbAg]_s} = \frac{K_2[Ag]_s}{2}. \quad (7)$$

The value of $K_2[Ag]_s$ is often termed in literature as the "enhancement factor". As can be seen from Eq. (7), this factor is equal to the double ratio of bivalently and monovalently bound antibodies. The ratio of bivalently and monovalently bound mAbs may be obtained from Eq. (6), when the values of K_{obs} for bAbs (K_1) and K_{obs} for parental mAbs are known.

2.10.6. Theoretically expected variations of kinetic parameters depending on the absence or presence of bivalent binding

The above-mentioned equations also allow to compare the kinetic parameters of the interaction of parental mAbs and bAbs with immobilized antigens. From Eq. (4), the observed kinetic association constant (k_{ass}) for bAbs is equal to k_1. k_{ass} for parental mAbs usually is taken to be independent of the valence of binding [16] and is equal to $2k_1$. The observed kinetic dissociation constant (k_{diss}) for bAbs is equal to k_{-1}. k_{diss} for parental mAbs may be obtained from Eq. (6):

$$k_{diss} = \frac{k_{-1}}{K_2[Ag]_s/2 + 1}, \quad (s^{-1}). \quad (8)$$

Consequently, the rate of dissociation of parental mAbs, which are able to bind bivalently, is significantly lower than the dissociation rate of bAbs. If bivalent binding is not possible, for some reason, then bAbs and mAbs will show the identical dissociation rates.

3. Results

3.1. Characterization of parental mAbs and bAbs

The analysed antibody panel included mAbs and bAbs specific to three different antigens: Mb ($M_r \approx$ 17,800), HRP ($M_r \approx 40{,}000$) and hIgG ($M_r \approx$ 160,000). BAbs (anti-Mb/HRP and anti-hIgG/HRP) were obtained by the fusion of anti-Mb hybridoma (clone 14D6) or anti-hIgG hybridoma (clone 75G5) with the same anti-HRP hybridoma (clone 36F9) [13,23]. The binding of antibodies with antigens adsorbed on a plastic was studied using the immunoenzymatic and radioimmune methods.

3.2. The analysis of the binding of the parental mAbs and bAbs with immobilized antigens using the immunoenzymatic method

The analysed antibody panel included anti-HRP mAbs (clone 36F9) and bAbs bearing anti-HRP binding sites. For this reason, we have excluded the use of HRP-labeled secondary antibodies. To register antibody binding, we have used biotinilated anti-mouse antibodies and avidin, conjugated with alkaline phosphatase. As all antibodies were of IgG1 subclass, it may be assumed that all bound antibodies were identically registered by the selected system. Binding curves (absorbance vs. antibody concentration) are shown in Figs. 2a, 3a and 4a. In addition, Table 1 presents the values of binding curves coefficients (the tangents of the slope of binding curves to abscissa) for mAbs and bAbs. Proceeding from the ratio of these coefficients (Table 1), it can be concluded that anti-Mb mAbs (clone 14D6) show 10 times higher binding with adsorbed Mb than monovalent anti-Mb/HRP bAbs (Figs. 2a). We have also observed the significant difference in the binding of anti-HRP mAbs and bAbs with immobilized HRP: mAbs show 7.5 times higher binding than bAbs do (Fig. 3a, Table 1). BAbs obtained from different tetradomas, but bearing the identical anti-HRP binding site (anti-Mb/HRP bAbs and anti-hIgG/HRP bAbs), show the identical binding with immobilized HRP (Fig. 3a). At the same time, anti-hIgG mAbs (clone 75G5) showed only 1.3 times higher binding with adsorbed hIgG than anti-hIgG/HRP bAbs (Fig. 4a, Table 1). Thus, the ratio of binding curve coefficients for mAbs and bAbs in case of immobilized Mb and HRP is significantly higher than 2. As follows from the theoretical background (see Section 2.10.4), this ratio is consistent with the proposition that some part of bound anti-HRP and anti-Mb mAbs is associated bivalently with the corresponding anti-

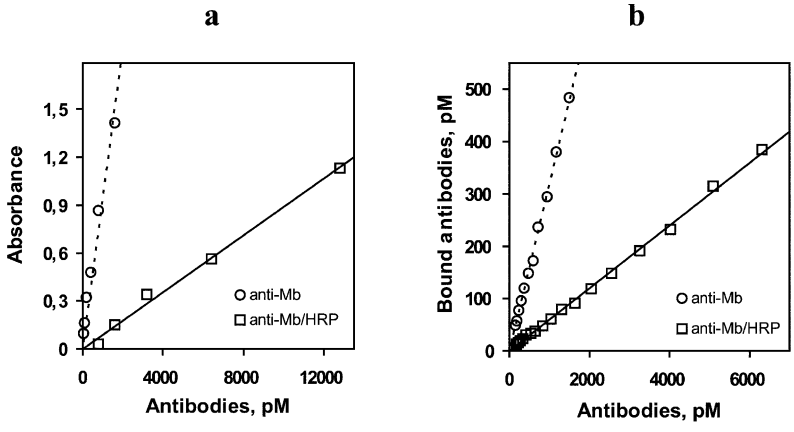

Fig. 2. Binding of parental monoclonal antibodies and bispecific antibodies to human myoglobin adsorbed on a solid phase. (a) Binding of anti-Mb mAbs (14D6) and anti-Mb/HRP bAbs (14D6 × 36F9) to immobilized Mb was measured by ELISA, using the biotinilated sheep anti-mouse IgG antibodies (second antibodies) and alkaline phosphatase conjugated to avidin (see Section 2.6). (b) Binding of anti-Mb mAbs (14D6) and anti-Mb/HRP bAbs (14D6 × 36F9) to immobilized Mb was measured by RIA, using ^{125}I-labeled mAbs or bAbs (see Section 2.9). (○---○)—anti-Mb mAbs 14D6; (□—□)—anti-Mb/HRP bAbs 14D6 × 36F9.

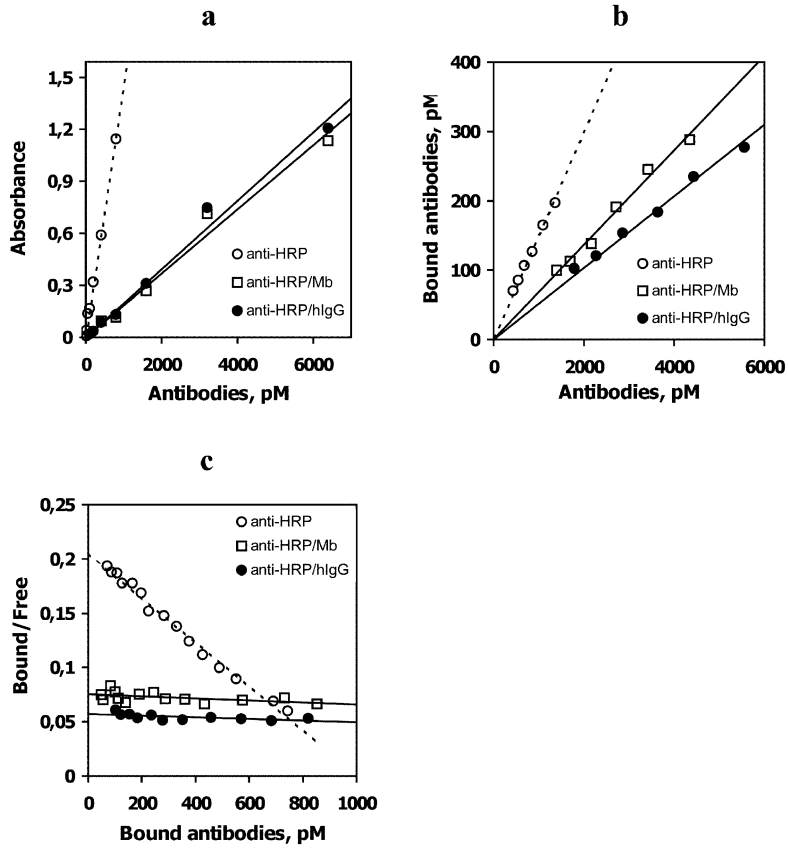

Fig. 3. Binding of parental monoclonal antibodies and bispecific antibodies to HRP adsorbed on a solid phase. (a) Binding of anti-HRP mAbs (36F9), anti-Mb/HRP bAbs (14D6 × 36F9) and anti-hIgG/HRP bAbs (75G5 × 36F9) to immobilized HRP was measured by ELISA, using the biotinilated sheep anti-mouse IgG antibodies (second antibodies) and alkaline phosphatase conjugated to avidin (see Section 2.6). (b) Binding of anti-HRP mAbs (36F9), anti-Mb/HRP bAbs (14D6 × 36F9) and anti-hIgG/HRP bAbs (75G5 × 36F9) to immobilized HRP was measured by RIA, using ^{125}I-labeled mAbs or bAbs (see Section 2.9). (c) Scatchard plots of "b". (○---○)—anti-HRP mAbs 36F9; (□—□)—anti-Mb/HRP mAbs 14D6 × 36F9; (●—●)—anti-hIgG/HRP mAbs 75G5 × 36F9.

gens. In contrast, for hIgG, the ratio of mAbs and bAbs binding curves coefficients is less than 2, suggesting the absence of bivalent binding of parental anti-hIgG mAbs with immobilized antigen (see Section 2.10.4).

In addition, we have studied whether anti-Mb/HRP bAbs can bind Mb and HRP simultaneously, in the situation when the equimolar mixture of both antigens (Mb and HRP) is adsorbed on the surface of a solid phase. In Fig. 5, binding curves of bAbs, obtained with the mixture of two antigens (Mb + HRP), are compared with binding curves for the same bAbs obtained with individually immobilized antigens (Mb or HRP). The total molar concentration of adsorbed proteins was the same in both experiments. As can be seen from these data (Fig. 5), bAbs show 3–6 times higher binding with the antigen mixture than with individual antigens. These data suggest the presence of bivalent binding of bAbs, when both antigens are simultaneously adsorbed on the plastic.

3.3. Radioimmunological analysis of the binding of the parental mAbs and bAbs with immobilized antigens

In these experiments, bAbs and mAbs were labeled with ^{125}I. Figs. 2b, 3b and 4b represent titra-

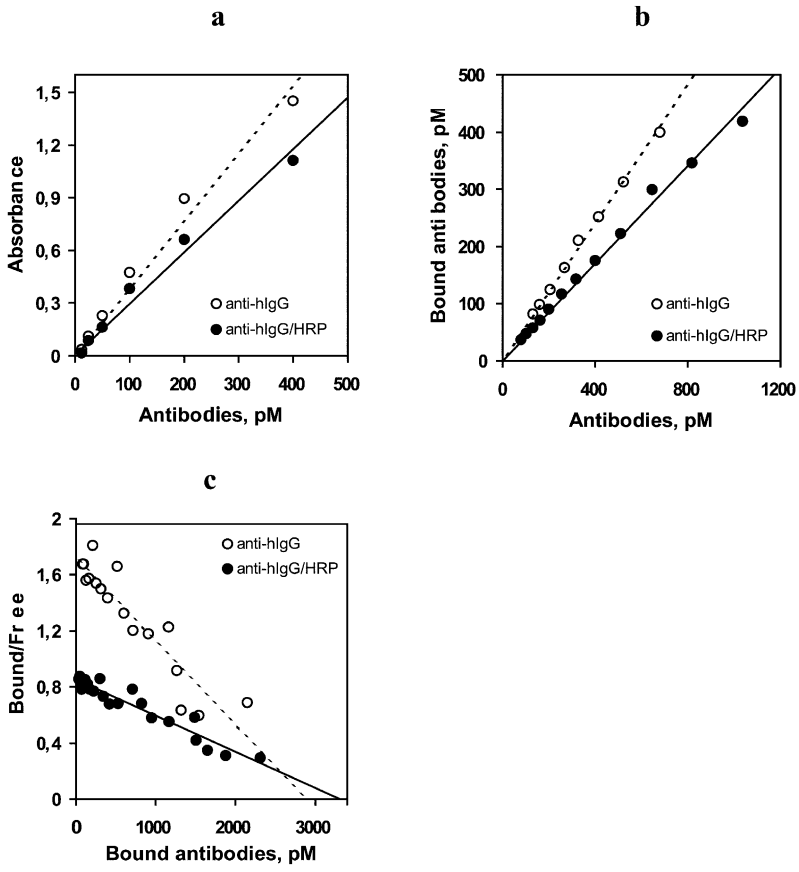

Fig. 4. Binding of parental monoclonal antibodies and bispecific antibodies to human IgG adsorbed on a solid phase. (a) Binding of anti-hIgG mAbs (75G5) and anti-hIgG/HRP bAbs (75G5 × 36F9) to immobilized IgG was measured by ELISA, using the biotinilated sheep anti-mouse IgG antibodies (second antibodies) and alkaline phosphatase conjugated to avidin (see Section 2.6). (b) Binding of anti-hIgG mAbs (75G5) and anti-hIgG/HRP bAbs (75G5 × 36F9) to immobilized IgG was measured by RIA, using ^{125}I-labeled mAbs or bAbs (see Section 2.9). (c) Scatchard plots of "b". (○---○)—anti-hIgG mAbs 75G5; (●—●)—anti-hIgG/HRP bAbs 75G5 × 36F9.

tion curves obtained with mAbs and bAbs (concentration of bound antibodies vs. total concentration of antibodies). Scatchard plots of the same binding experiments are presented in Figs. 3c and 4c. It should be noted that Scatchard analysis of binding curves requires a correct determination of concentrations of bound and free antibodies. Bearing in mind this circumstance, we have subjected all antibodies after iodination to affinity purification on antigen-Sepharose columns. Therefore, it may be assumed that after affinity purification we obtained IgG fractions in which all ^{125}I-bound antibodies were immunologically active. This fact seems rather important for Scatchard analysis, since some of the antibody molecules may be inactivated during iodination [32]. The concentration of immunologically active antibodies after affinity purification was measured as described in Section 2.8.

The comparison of titration curves, presented in Figs. 2b, 3b and 4b, allows to reveal the significant difference in binding of mAbs and bAbs with immobilized HRP and Mb. Proceeding from the ratio of calibration curve coefficients (Table 1), it may be concluded that ^{125}I-labeled anti-Mb mAbs show 5.3 times higher binding with immobilized Mb than ^{125}I-bAbs (Fig. 2b). For ^{125}I-labeled anti-HRP mAbs and ^{125}I-labeled bAbs with specificity anti-Mb/HRP and anti-hIgG/HRP, this ratio (with respect to HRP)

Table 1
The coefficients of titration curves (the tangents of the slopes of binding curves to abscissa) obtained with parental mAbs and bispecific antibodies

Method	Antibody specificity	Immobilized antigen	Figure	Binding curve coefficient
ELISA	anti-Mb	Mb	2a	0.00095 ± 0.00006
	anti-Mb/HRP	Mb	2a	0.00009 ± 0.00001
	anti-HRP	HRP	3a	0.00145 ± 0.00003
	anti-Mb/HRP	HRP	3a	0.00019 ± 0.00001
	anti-hIgG/HRP	HRP	3a	0.00020 ± 0.00001
	anti-hIgG	hIgG1	4a	0.0038 ± 0.0002
	anti-hIgG/HRP	hIgG1	4a	0.0029 ± 0.0001
RIA	anti-Mb	Mb	2b	0.319 ± 0.003
	anti-Mb/HRP	Mb	2b	0.060 ± 0.001
	anti-HRP	HRP	3b	0.149 ± 0.002
	anti-Mb/HRP	HRP	3b	0.068 ± 0.001
	anti-hIgG/HRP	HRP	3b	0.052 ± 0.001
	anti-hIgG	hIgG1	4b	0.603 ± 0.006
	anti-hIgG/HRP	hIgG1	4b	0.426 ± 0.007

constitutes 2.2 and 2.9, correspondingly (Fig. 3b, Table 1). ^{125}I-anti-hIgG mAbs showed 1.4 times higher binding with immobilized hIgG (Fig. 4b, Table 1) than the corresponding bAbs (anti-hIgG/HRP). As a whole, the data obtained in solid-phase RIA confirm the conclusions made on the basis of ELISA. The relatively high ratio of binding curve coefficients for mAbs and bAbs with respect to immobilized Mb and HRP (>2) allows to suggest the presence of bivalent binding of anti-Mb and anti-HRP mAbs with antigens adsorbed on the surface of immunoplates. For immobilized hIgG, the ratio of binding curve coefficient for bivalent and monovalent antibodies is less than 2 (1.4); this, as has been shown in theoretical Section 2.10.4, is not consistent with the proposition of the ability of anti-hIgG mAbs to bivalently bind the immobilized antigens. It should be noticed, that in solid-phase RIA, the differences between mAbs and bAbs were not so strong as in ELISA.

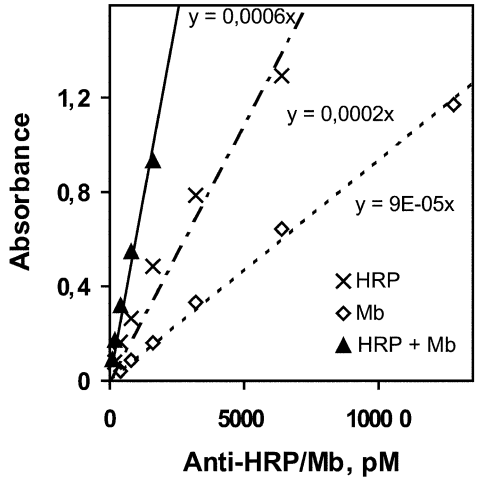

Fig. 5. Binding of antibodies with double specificity to human myoglobin and HRP (14D6×36F9) to immobilized antigens, taken individually or in mixture. (×-·-×)—the wells of immunoplates were coated with HRP (10 μg/ml in 0.05 M carbonate buffer, pH 9.5); (◇---◇)—the wells of immunoplates were coated with Mb (10 μg/ml in 0.05M carbonate buffer, pH 9.5); (▲—▲)—the wells of immunoplates were coated with the equimolar mixture of two antigens—Mb and HRP (6.8 μg/ml of HRP and 3.2 μg/ml of Mb in 0.05 M carbonate buffer, pH 9.5). Binding of bAbs with immobilized Mb and HRP was measured by ELISA, using the biotinilated sheep anti-mouse IgG antibodies (second antibodies) and alkaline phosphatase conjugated to avidin (see Section 2.6).

Scatchard plots of the same binding experiments (Figs. 3c and 4c) were used to determine the observed equilibrium binding constants (K_{obs}). It should be reminded that K_{obs} is equal to the value of the tangent of the slope of the best-fit line through the data. The values of K_{obs} for mAbs and bAbs, obtained from the corresponding Scatchard plots, are presented in Table 2. As seen from Table 2, K_{obs} for parental mAbs, specific to HRP is 38 times higher K_{obs} for anti-HRP sites of bAbs with double specificity to HRP and hIgG, and 21 times higher K_{obs}

Table 2
Observed equilibrium constants (K_{obs}) for the binding of parental monoclonal antibodies and bispecific antibodies to antigens adsorbed on a solid phase

Cell line	Antibody specificity	Immobilized antigen	K_{obs}, M^{-1}
36F9	Anti-HRP	HRP	$(2.0\pm0.1)\times10^8$
36F9×14D6	Anti-Mb/HRP	HRP	$(9.6\pm0.8)\times10^6$
36F9×75G5	anti-hIgG/HRP	HRP	$(5.3\pm0.5)\times10^6$
75G5	Anti-hIgG	hIgG1	$(5.9\pm0.6)\times10^8$
36F9×75G5	anti-hIgG/HRP	hIgG1	$(2.6\pm0.2)\times10^8$

for bAbs with specificity anti-Mb/HRP. It should be noted that the difference in the K_{obs} for the anti-HRP sites of two kinds of bAbs (anti-hIgG/HRP and anti-Mb/HRP) does not exceed the scatter common for such experiments [14,16–18]. The results of Scatchard analysis were also applied in order to determine the percentage of monovalently and bivalently bound parental mAbs on the surface of a solid phase. Eqs. (6) and (7) (see theoretical Sections 2.10.2 and 2.10.5) allow to calculate the ratio of bivalently and monovalently bound mAbs, using the experimentally obtained K_{obs}. For anti-HRP mAbs, this ratio constitutes 9 or 18, depending on whether the data for anti-Mb/HRP bAbs or anti-hIgG/HRP bAbs was used in the calculations of the affinity of monovalent binding. Therefore, for the given antigen density on the surface, and in the given range of antibody concentrations in the incubation mixture, about 90–95% of all bound parental anti-HRP mAbs are associated bivalently, and only 5–10% are associated monovalently with adsorbed HRP. In the case of adsorbed hIgG, the K_{obs} of parental mAbs was only 2.3 times higher K_{obs} for anti-hIgG site of bAbs (Table 2). In this case, the ratio of K_{obs} for mAbs and bAbs is consistent with the above-made conclusion about the absence of bivalent binding of parental mAbs with adsorbed hIgG. Indeed, theoretically, in the absence of bivalent binding, the K_{obs} of mAbs should be two times higher K_{obs} for bAbs, due to the statistical factor of 2 (see Section 2.10.3). Evidently, the deviation of experimental K_{obs} values for anti-hIgG mAbs from theoretical value does not exceed the scatter common for the method. The data on Scatchard analysis of antibody binding with Mb are not presented, as we did not obtain the proper Scatchard plots in any of the three independent experiments (the scatter was too large).

4. Discussion

With the widespread use of mAbs in solid-phase immunoassays and in cell targeting, a need has arisen for a better understanding of the mechanisms governing the interactions of antibodies with immobilized antigens. Theoretical aspects of this problem have been considered in a number of studies [14–16]. The main principles of the accepted model of bivalent binding in solid-phase systems, concerning the subject of our work, are summarized in the theoretical Section 2.10. As follows from this model, the ability of mAbs to bind bivalently to immobilized antigens may produce strong enhancement effect on the strength of antigen–antibody interaction. This enhancement effect in an experimental system of interest may be evidenced and quantitatively estimated in equilibrium binding experiments with knowledge of the true affinity of monovalent binding of IgG antibody. This approach was applied in a number of experimental studies on the mechanisms of binding of mAbs with solid-phase antigens [14–21]. Moreover, the common way to measure the intrinsic affinity of monovalent binding of IgG antibodies to immobilized antigens was the use of their F(ab) fragments [14–17]. However, it should be borne in mind that this approach might have some disadvantages, due to the differences in the structure of F(ab) fragments and intact antibodies. As shown in some studies, the Fc region (which is absent in F(ab) fragments) is able to influence the antibody binding characteristics, possibly by influencing antibody flexibility [33]. It should be noted that the affinity of monovalent binding of bivalent antibody with a soluble antigen might be easily measured in equilibrium binding experiments in solution. However, the immobilization on a solid phase (the surface of immunoplates) may result in a partial denaturation of the protein, modifying its binding property [34]. Therefore, the absolute values of affinity constants measured in solution are not valid for solid-phase experiments.

The distinctive feature of the present study on the interaction of mAbs with solid-phase antigens is the use of biologically produced bAbs to measure the

intrinsic affinity. The paper reports the results of the comparative analysis of the ability of three mAbs of different specificity (anti-Mb, anti-hIgG and anti-HRP) and bAbs derived thereof to bind antigens adsorbed on a surface of immunoplates. A bAbs molecule secreted by tetradoma cells is composed of two different halves of parental mAbs. According to this scheme, bAbs and parental mAbs should have the same antigen-binding site structure, and, consequently, the same intrinsic affinity. In fact, in one of our recent works we have confirmed that bAbs anti-hIgG/HRP (used in the present study) and their parental mAbs have identical affinity constants with respect to both antigens, as defined by Scatchard plot analysis of the equilibrium binding data for the solution [5]. Other authors also demonstrated the identity of antigen-binding sites of mAbs and bAbs derived thereof [4]. In general, the production of bAbs with altered affinity of antigen-binding sites (i.e., due to the "incorrect" association of H and L chains in bAbs shoulders) is considered to be a rare event [3], though such cases have been reported [7]. Since all mAbs analysed in the present work were of IgG1 subclass, bAbs derived thereof had the same structure of their Fc region as the bivalent antibodies. Theoretically, the use of bAbs retaining the structure of the intact IgG molecule to measure the intrinsic affinity may provide more correct estimation of the influence of bivalence on antigen–antibody binding in heterogeneous systems, if compared with the experimental models using F(ab)s. In the present study, the comparison of equilibrium binding data for mAbs and bAbs, obtained in two methods (ELISA and solid-phase RIA), with the predictions of the bivalent binding model allowed to reveal the strong enhancement effects due to bivalent binding of anti-HRP and anti-Mb mAbs to immobilized antigens. At the same time, the observed differences in the binding curves' parameters for anti-hIgG mAbs and corresponding anti-hIgG/HRP bAbs (concerning anti-hIgG arm) were not enough to testify the bivalent binding and had to be attributed to the statistical factor of two binding sites per bivalent antibody molecule.

Several reasons could account for the lack of bivalent interaction of anti-hIgG mAbs with immobilized antigen molecules. The trivial reason is that the antibody does not contain two active binding sites. For example, binding sites may be inactivated during the affinity purification of antibodies. However, as antibody activity was specially controlled in our work during the purification (see Section 2.3), this possibility can apparently be excluded. Binding sites also may be inactivated during iodination. But evidently, this could not be the reason for the observed absence of bivalent binding in ELISA. It may be also proposed that the density of antigen is too low to permit bivalent binding. This reason also seems hardly probable, as we did not observe this effect with other antibodies, using the same concentrations of antigens in solution to saturate the immunoplates. At last, it may be suggested that the observed differences in the behaviour of different mAbs depend on steric factors. It has been recognized previously that avidity depends strongly on whether the structure of antigen allows bivalent antibody binding [35]. In this connection, it should be noticed that hIgG has a "stick" structure, while Mb and HRP have a globular structure. Our study confirms previous observations (obtained with F(ab) fragments) [16–21] that though some antibodies can bind bivalently, others bind monovalently in the same experimental conditions. Accordingly, the degree of difference between different bAbs and their corresponding intact native molecules should be expected to vary markedly from antibody to antibody, as observed in our work. In the earlier study of Dower et al. [16], it was shown that antibodies to different epitopes of the same cell surface antigen might exhibit different modes of binding (bivalent or monovalent).

In addition, it should be noticed that the differences in binding curves for anti-HRP and anti-Mb mAbs and corresponding bAbs were more pronounced in ELISA than in RIA (Fig. 2a and b, Fig. 3a and b; Table 1). To explain this effect, it should be borne in mind that bAbs might be more strongly dissociated during the incubations than bivalently bound anti-HRP and anti-Mb mAbs (see Section 2.10.6). Evidently, the effect of dissociation should be more visible in the multi-step method (in our work, it is the ELISA method) than in a one-stage procedure (RIA method). In the absence of bivalent binding (anti-hIgG mAbs), the ratio of binding curve parameters for bAbs and mAbs was practically the same in ELISA and RIA (Fig 4a and b; Table 1), apparently because bAbs and mAbs had the same dissociation rates in this case.

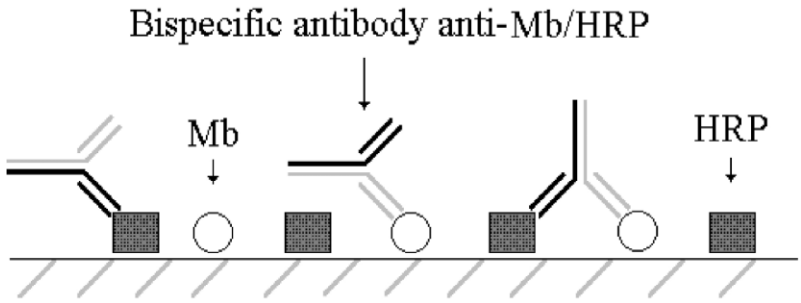

Fig. 6. Binding of antibodies with double specificity to human myoglobin and HRP to antigens simultaneously adsorbed on a solid phase. If both antigens (Mb and HRP) are simultaneously immobilized on a surface of immunoplates, the monovalently and bivalently bound states of the antibody co-exist in equilibrium with one another on the surface of a solid phase. In the bivalent state, both antigen-binding sites of bAbs simultaneously bind two antigens that should result in the increased avidity of the binding.

In our work, the enhancement effect due to the bivalent interaction was also evidenced in the experiments on comparison of binding of anti-Mb/HRP bAbs with individually immobilized antigens, and their mixture. A model for the effect is shown in Fig. 6. These data are in line with the earlier study of Wong and Colvin [36], in which two bAbs with double specificity against different T cell surface antigens were produced. These bAbs promoted complement-mediated lysis of target cells that bore both surface antigens 25 to 3125 times more efficiently than of cells expressing only one of the antigens.

Milstein and Cuello [3] in their pioneer work on the production of bAbs by cell fusion first showed that antibodies with dual specificity to antigen and enzyme (anti-somatostatin/HRP) might be effectively used in immunohistochemistry. In a number of later studies, the replacement of traditional enzyme–antibody conjugates with tetradoma-produced bAbs, bearing binding sites to the antigen and the enzyme, has been claimed to be an advantageous approach for the development of high performance heterogeneous immunoassays and immunohistochemistry [9–12]. On the contrary, the results of our study give evidence that bAbs show strongly reduced avidity in heterogeneous systems, if compared with mAbs binding bivalently (anti-Mb and anti-HRP mAbs). In general, the reported data remind that multivalency is the advantage of the natural antibody, by which great increase in functional affinity may be achieved. This may be very important for the variety of applications of antibodies in vitro and in vivo. For example, in our recent study we have compared the efficiency of the traditional mAb-enzyme (HRP) conjugate and bAbs, carrying anti-Mb and anti-HRP sites, in antigen-capture ELISA for human Mb [13]. It has been shown that parental mAbs were more effective than bAbs, regarding sensitivity and resolution of the method [13]. In the past 10–15 years, important advances have been made in the design, selection and production of the new type of engineered antibodies—so-called mini-antibodies or recombinant single-chain Fv fragments [37–39]. In particular, some authors reported the attempt to produce tetravalent bispecific single-chain antibody [40,41]. Theoretically, this approach to produce the bispecific reagents with higher avidity seems to be very promising. However, in practice, it is still very difficult to obtain tetravalent single-chain bAbs with improved binding characteristics [40,41]. Therefore, this approach requires further elaboration.

We suppose that the results of our study warn against using biologically produced bAbs as labeled antibodies in solid-phase systems without accounting the avidity effects. The process of obtaining the producer antibody clones is rather laborious and requires high skill. Problems can also occur during bAbs purification. But this approach hardly can provide the significant gain in assay performance, if parental mAbs are able to bind bivalently.

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