М Е Т О Д И

NOVEL AND SIMPLE ELISA-BASED METHOD FOR ANTIBODY AFFINITY DETERMINATION

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В статті запропоновано метод визначення афінності антитіл за допомогою ELISA, що грунтується на нових координатах. Запропонований підхід є надзвичайно простим і разом з тим він позбавлений деяких недоліків, властивих відомим раніше і широко використовуваним методам B. Friguet et al. (1985). У статті наведено приклади застосування нового підходу для визначення афінності антитіл з використанням як теоретичних, так і експериментальних кривих зв'язування антитіл з іммобілізованим на імунологічних платах антигеном. Теоретичні та експериментальні криві зв'язування антитіл розглянуто за умови, що ці антитіла є частково заблоковані антигеном, який знаходиться в розчині разом з антитілами, причому концентрація вільних паратопів антитіл та концентрація комплексів антиген–антитіло є в стадії динамічної рівноваги.

К л ю ч о в і с л о в а: антиген, антитіло, взаємодія антиген–антитіло, афінність.

ELISA-based methods for the evaluation of antibody affinity are broadly used because of their simplicity and rather quickness. No less important features of these methods are that they allow using small amount of antibodies and an antigen which purification is not necessary. For these reasons the method proposed by B. Friguet et al. [1], which described a relatively simple approach for antibody affinity determination by ELISA, became very popular and widespread [2]. The method is based on the idea of measuring the fraction of free antibodies in antigen-antibody (Ag-Ab) mixtures by ELISA if the state of dynamic equilibrium has been achieved.

Earlier it was already shown by us [3–5] that the method of B. Friguet et al. has some disadvantages. Among these disadvantages there are the usages of the reverse concentrations of the antigen for the construction of appropriate plots, somewhat higher complexity of the equations than it could be applied for such purposes, and that the way for obtaining these formulas is not optimal. Several new equations were suggested by us [3–5], which are more convenient, simpler, and should give more precise results for antibody affinity evaluation than B. Friguet et al. equations.

In the present paper we suggest one more equation which is not only very simple, but its application presumes usage of the direct values of antigen concentration for plotting the binding curves, and such approach allows to evaluate the antibody affinity more precisely than the equations suggested earlier by others [1]. Advantages of equations for antibody affinity evaluation, suggested by us earlier and in the present paper, were proved more precise not only theoretically but also using experimental binding curves.

Materials and Methods

Bovine serum albumin (BSA), hen's egg albumin (OVA) and IgG monoclonal antibodies (mAbs) against OVA (mAbs anti-OVA) were purchased from «Sigma», USA. Polystyrene flat-bottomed 96-well plates for ELISA («Dynatech», Sweden) were covered by OVA as follows. Solution of OVA (30 μg/ml) in 1.5% NH_4HCO_3 (pH 9.0–9.5) was incubated for 16–18 h at 4 °C, removed, and the plates were thoroughly washed with distilled water before using.

A suitable dilution of the stock solution of anti-OVA mAbs was determined in preliminary experiments and it was about 1:400 000. All experiments with mAbs were carried out in phosphate buffered saline, pH 7.2 + 0.05% Tween 20 (TBS) + 1%BSA (BSA-TBS). OVA was dissolved also in BSA-TBS and aliquots with different concentration of OVA (from 1.0×10^{-8} to 5.0×10^{-7} M) were prepared. These aliquots (0.3 ml) of antigen were mixed with equal volumes of mAbs, prediluted in BSA-TBS 1:200 000, and the samples were incubated at room temperature for about 18 h to reach equilibrium between free mAb paratopes and mAb-OVA complexes.

After this the solutions of mAb-OVA mixtures were placed into 96-well plates precoated with OVA (100 μl/well, four wells per each variant), incubated 40 min at room temperature (about 25 °C) on a shaker and the amount of IgG bound to immobi-

lized antigens was determined by standard ELISA. Briefly, the plates after removing Ag-Ab solutions and washing were incubated with goat anti-mouse IgG labeled with horseradish peroxidase («Sigma», USA), and the peroxidase reactivity was revealed with *o*-phenylendiamine (0.5 mg/ml in phosphate buffer, pH 5.0) + 0.03% H_2O_2. The reaction was stopped with 2 M H_2SO_4 and the optical density (OD) was read at 490 nm.

In preliminary experiments we had found that the dilution of anti-OVA mAbs stock solution at least to 1:400 000 and more led to the proportional decrease of OD of plate wells, obtained by ELISA. This means that the values of OD, which are proportional to the amount of mAbs bound to immobilized antigen, at such and higher dilutions are proportional to the concentration of free mAbs in the studied mAb-OVA mixtures. For this reason we used anti-OVA mAbs pre-diluted 1:400 000 in all experiments. This allowed us to use the values of experimentally obtained OD in all further calculations instead of the real values of mAbs concentrations.

Theory

We have showed earlier [4] that if the total concentration of antigen, l_i, is much higher than the concentration of Ag-Ab complex then the mass action law in terms of the values measured by ELISA can be expressed as

$$K_d = \frac{l_i A_i}{A_0 - A_i} \quad (1)$$

where K_d is the dissociation constant of Ag-Ab complex, A_i and A_0 are the OD of the immunologic plate wells, which are proportional to the concentration of free antibodies at Ag concentration either l_i or zero, respectively.

It was also shown that simple algebraic transformations of equation (1) allow obtaining at least eight different equations suitable for the evaluation of the dissociation constant, K_d, for Ag-Ab interaction or its reversible value, the so called affinity constant, $K_a = 1/K_d$,. These equations are the following:

$$l_i A_i = K_d \times (A_0 - A_i) \quad (2)$$

$$\frac{l_i}{A_0 - A_i} = \frac{K_d}{A_i} \quad (3)$$

$$\frac{A_0 - A_i}{l_i} = K_a \times A_i \quad (4)$$

$$\frac{A_0 - A_i}{l_i} = K_a \times [A_0 - (A_0 - A_i)] \quad (5)$$

$$\frac{A_0 - A_i}{A_0} \times \frac{1}{l_i} = \frac{1}{K_d} \times \left(1 - \frac{A_0 - A_i}{A_0}\right) \quad (6)$$

$$\frac{A_0 - A_i}{A_i} = K_a \times l_i \quad (7)$$

$$\frac{A_i}{A_0 - A_i} = \frac{K_d}{l_i} \quad (8)$$

$$\frac{A_0}{A_0 - A_i} = 1 + \frac{K_d}{l_i} \quad (9)$$

It should be noted that equations (6) and (9) were also obtained earlier by B. Friguet et al., but these authors used much more complicated methods for their derivation. To obtain equations (6) and (9) these authors used algebraic transformation of G. Scatchard [7] and I. M. Klotz [8] equations, respectively.

In addition to the listed above equations (2)-(9), one more equation can be easily derived using equation (7), namely:

$$\frac{A_0}{A_i} = 1 + K_a \times l_i \quad (10)$$

Then, according to equation (10), the tangent of the slope of the linear relation between l_i and the values of A_0/A_i is equal to the value of K_a. One may see that this equation is very simple and it allows to compose the appropriate binding curve using not reverse but direct concentrations, l_i, of the competing antigen. As it was shown by us earlier [4], the use of direct concentrations of antigens could be very important for the precise determination of K_a values. Thus, equation (10), because of its simplicity and because the application of direct (not reverse) concentration of competing antigen, l_i, should be, obviously, more convenient and more precise for the evaluation of antibody affinity by ELISA than the suggested earlier B. Friguet et al. equations.

Results and Discussion

In order to estimate which of the above equations (2)–(10) is more suitable and precise for the antibody affinity evaluation let us consider at first the theoretical binding curves obtained according to each of these equations. To obtain these curves the theoretical values of A_i were calculated by equation (11) and some error was included deliberately in the obtained results by randomized function. This has been done to simulate a real situation, which occurs in experiments on antibody affinity determination, because experimental results usually have some experimental error. The use of these "non precise" theoretical curves would allow us to reveal which of the suggested nine formulas gives better precision in K_a determination and which of them are most dependent on the accuracy of experimental measurements.

To calculate the value of the concentration of

Ag-Ab complex, c_i, for putative monovalent antibodies (antibody concentration, $r_0 = 1\times10^{-9}$ M; antibody affinity $K_d = 1\times10^{-9}$ M) with appropriate antigen (concentration range $l_i = 1.25\times10^{-9} - 8.0\times10^{-8}$ M) we used the following equation:

$$c_i = \frac{K_d + r_0 + l_i}{2} + \sqrt{\left(\frac{K_d + r_0 + l_i}{2}\right)^2 - r_0 l_i} \qquad (11)$$

where r_0 is the total concentration of antibody paratops (see [4]). Knowing the value of c_i it is possible to determine the concentration of free (non-blocked by antigen) paratops, r_i, by subtracting c_i from r_0. Because of proportionality between the values of r_0 and A_0, and between r_i and A_i, we can use the obtained values of r_0 and r_i instead of the values of A_0 and A_i in the equations (2–10) and to compose the appropriate binding curves (Fig. 1, *A–I*).

To simulate a real experimental situation the precise theoretical values of A_i obtained by this way were changed either by adding or subtracting random values (not more then ±10% of A_i) generated by randomized function of program Excel. Then, the obtained "non-precise" values of A_i were used for constructing the appropriate binding curves in coordinates suggested by one of the equations (2)–(10). The slopes of trend lines of these binding curves, determined by program Excel, were equal to the values of K_a (or $1/K_a = K_d$). The values of affinity, K_a, determined by this way using the equations (2)–(10) and the values of the function dispersion, R^2, are presented in Table 1.

One may see that the highest precision of K_a determination was obtained when equations (3), (7), and (10) were used. In addition, the minimal dispersion of the data was also obtained when these equations were applied. These results demonstrate that equations (3), (7), and (10) are most suitable for antibody affinity determination. On the other hand, the usage of equations suggested earlier by B. Friguet et al. (1985), namely equations (6) and (9), resulted in the obtaining almost the biggest deviation from the true value of K_a, which was equal for the given case to 1.0×10^9 M^{-1}. In fact, the values of K_a obtained by equations (6) and (9), were almost two times smaller than the right values, while they were very close to the theoretical values if equation (6) or (9) were used for their evaluation. The spread of the data was also higher for the case of equations (6) and (9) in comparison with the spread of the data when equations (3), (7) or (10) were used.

It is worth to underline that these results clearly demonstrate also that the better precision in K_a evaluation was obtained if direct values of antigen concentration, l_i, were put on the abscissa instead of the reverse values. In contrast, when the reverse values of l_i, as it was if equations (6) or (9) were used, this leads to the increase of the spread of the data and to the decrease of the accuracy of K_a determination. Thus, application of equations (3), (7), and (10) allows to get much more precise values than the use of equations (6) or (9), which were suggested earlier [1] and were broadly used till now.

At the same time, it is necessary to keep in mind that all the above considered approaches do not take into account the fact that antibodies are not monovalent but are, at least, bivalent ones. If bivalent antibody, which is semi-blocked by soluble antigen, can bind to immobilized antigen and can be determined in ELISA as free antibody, then the mistake in a real value of K_a, determined in this way, will be even higher than it was obtained above. Thus, using any of equations (2)–(10) for antibody affinity evaluation one has to remember that they were obtained for putative monovalent antibodies.

Let us consider now an example of K_a determination using not theoretical but experimental binding curves. The experiment was carried out with anti-OVA monoclonal antibodies and OVA from «Sigma». Solutions of OVA were used for covering the plates and for the reversible binding to mAbs in solution. The amount of free mAbs in mixtures with different concentrations of OVA, after the equilibrium of the reactions were achieved, was determined with ELISA and the obtained results are presented in Fig. 2. If the values of A_i are proportional to the amount of free antibodies in Ag-Ab mixture, then we can use these values for plotting the appropriate binding curve in coordinates suggested by equations (2)–(10).

As far as the above data have shown that the most precise values of K_a could be obtained using equations (3), (7), and (10) just these equations together with the equations (6) and (9) suggested earlier by B. Friguet et al. we applied for affinity determination of anti-OVA mAbs in the experiment. It is obvious that equations (7) and (10) should give the same values of affinity determination because their

Table 1. Values of antibody affinity, K_a, determined using equations (2)–(10) and theoretical values of A_i, calculated by equation (11) and distorted by randomized function up to ±10% of A_i

The number of used equation	2	3	4	5	6	7	8	9	10
K_a ($\times10^9$), M^{-1}	2.17	1.02	0.58	0.53	0.53	0.97	0.53	0.53	0.97
R^2	0.78	1.00	0.97	0.97	0.97	1.00	0.99	0.99	1.00

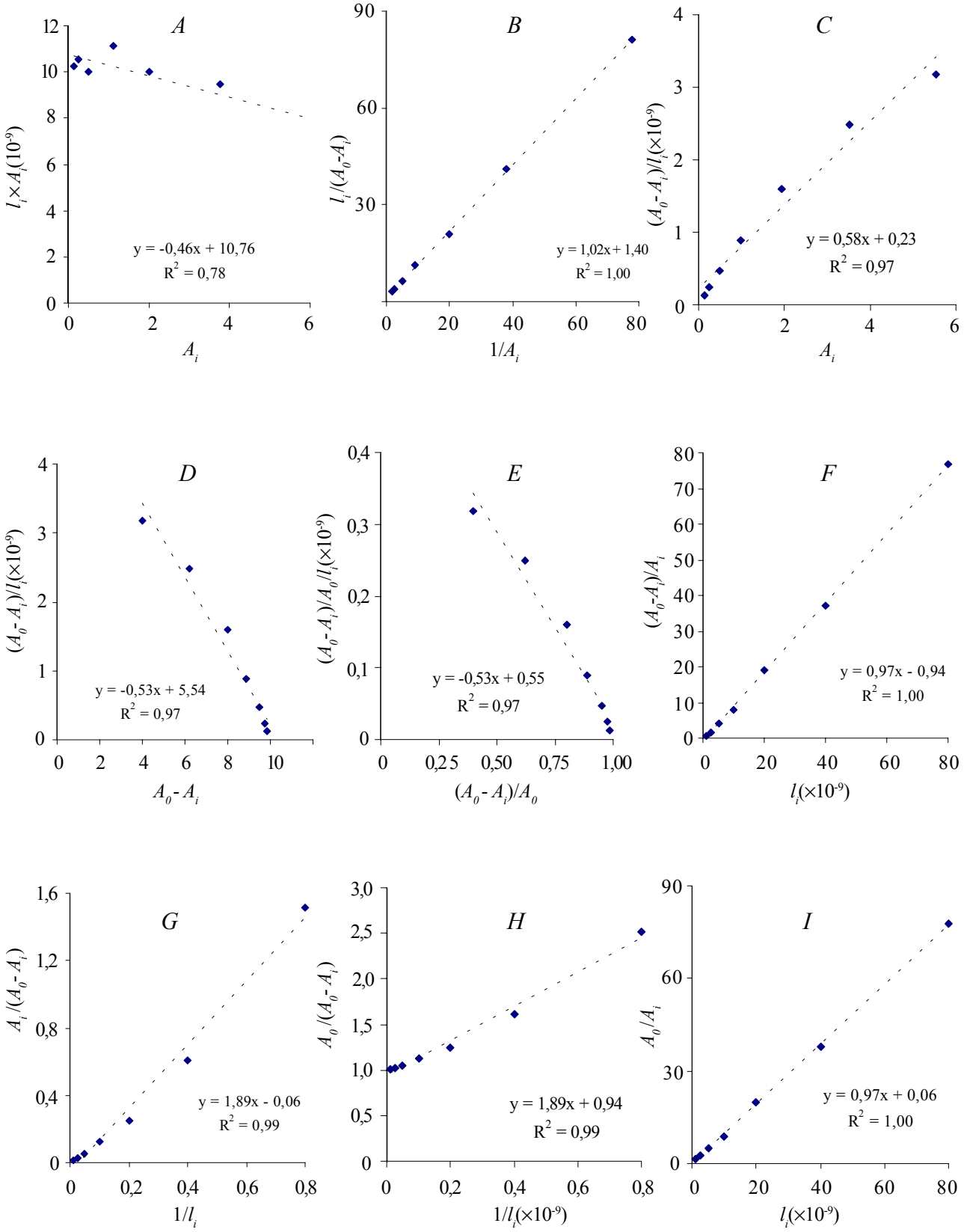

Fig. 1. Graphical representations of Eqs (2)-(10) by simulated binding curves for hypothetical monovalent antibody: A − Eq. (2), B − Eq. (3), C − Eq. (4), D − Eq. (5), E − Eq. (6), F − Eq. (7), G − Eq. (8), H − Eq. (9), I − Eq. (10). Theoretical values of A_i were calculated for the following parameters of antigen-antibody interaction: $K_a = 1{,}0\times10^{9}$ M^{-1}; $A_0 = 1$; $r_0 = 1{,}0\times10^{-9}$ M; $l_i = 0{,}125\times10^{-8} - 8{,}0\times10^{-8}$ M.

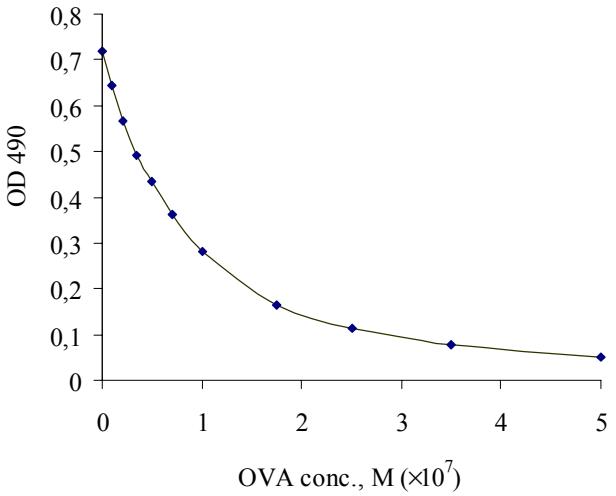

Fig. 2. Effect of the concentration of soluble OVA on the binding of anti-OVA mAbs to immobilized OVA if soluble OVA is in a mixture with anti-OVA mAbs and the state of equilibrium between free and occupied antibodies is achieved.

use gives similar plots (like equations (8) and (9)), which differ only in the shift of binding curves by unity in ordinate axis. The experimental data of Fig. 2 were used for the plotting of binding curves (Fig. 3) according to equations (3), (6), (9), and (10), namely in coordinates $l_i/(A_0\text{-}A_i)$ versus $1/A_i$, $(A_0\text{-}A_i)/l_iA_0$ versus $(A_0\text{-}A_i)/A_0$, $A_0/(A_0\text{-}A_i)$ versus $1/l_i$, and A_0/A_i versus l_i, respectively.

The obtained values of K_a for the interaction between anti-OVA mAbs and OVA are presented in Table 2. It could be seen that the use of equations (6) and (9) again yields more than two times less values of K_a than the use of equations (3) or (10). Although now the precise value of the affinity is not known in contrast to the former example when we considered the theoretical curves, remembering the former results we can suppose that the values of K_a obtained by equations (3) and (10) are more precise than two-times-less values obtained by B. Friguet et al. methods.

T a b l e 2. Values of antibody affinity, K_a, for anti-OVA mAbs determined by equations (3), (6), (9), and (10) using experimental values of A_i from Fig. 2, using the plots of Fig. 3

The number of used equation	K_a (×10^7), M^-1	R^2
3	2.96	0.994
6	1.09	0.989
9	1.19	0.998
10	2.66	0.988

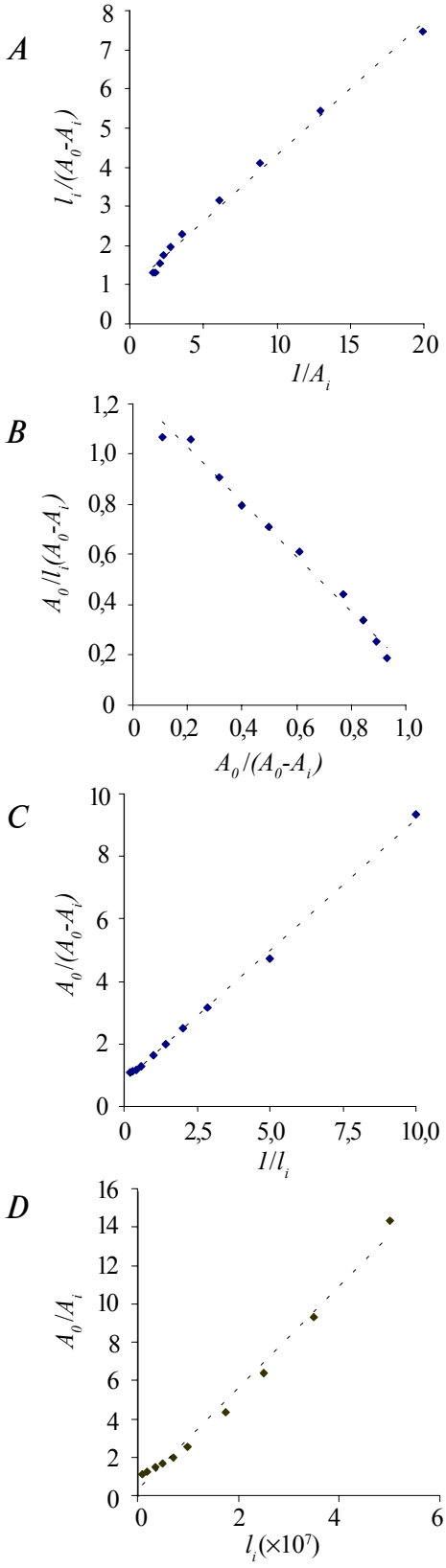

Fig. 3. Representation of the data from Fig. 2 in appropriate coordinates according to A – Eq. (3), B – Eq. (6), C – Eq. (8), and D – Eq. (10). The slopes of the trend lines for the obtained relations give the values of K_d or K_a.

Really, if more complex methods of K_a determination, which was described by us earlier [4, 6] and which takes into account the fact of bivalency of mAbs, were applied, then the value of the determined K_e will increase up to 5.3×10^7 M^{-1} (not shown). This shows us that the application of equations (3) and (10) yields much more precise results in comparison to the suggested earlier by B. Friguet et al. equations (6) and (9). In addition, equations suggested by us are simpler and give less dispersion of the data than the equations of B. Friguet et al. All this demonstrates the advantages of our equations (3) and (10) over Friguet's equations (6) and (9) for the antibody affinity evaluation. However, it should be kept in mind that the approaches considered in the present paper are simplified ones, which do not take into account the fact that the antibodies are at least bivalent reagents and not monovalent, as it was supposed by the used theory. If more precise affinity determination is necessary, then more complex approaches should be used, which were described by us earlier [4, 6].

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S u m m a r y

New coordinates for antibody affinity determination by ELISA are suggested. The suggested approach is very simple but at the same time it is more convenient and is deprived of the drawbacks inhered in the earlier suggested methods. The examples of antibody affinity determination by the suggested methods for both simulative and experimental binding curves are considered. It was demonstrated that the suggested methods allow getting more precise values of antibody affinity.

K e y w o r d s: antigen, antibody, antigen-antibody interaction, affinity.

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