Simulation of Competitive Radioimmunoassay Using Spreadsheet Program

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表計算シートを活用する競合型ラジオイムノアッセイのシミュレーション

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Worksheet templates have been prepared for use in developing novel immunoassay systems. One is for drawing Scatchard plots to calculate the affinity constant (Ka) from experimental data by least squares regression. The other is to construct the theoretical standard curve in competitive radioimmunoassay (RIA) using the dissociation constant (Kd=1/Ka). The former is useful to characterize antibodies for screening. The latter can be used to estimate the theoretical detection limit of competitive RIA, for comparison in assessment of developing noncompetitive hapten immunoassay system. The evaluation of these worksheets was carried out by applying them to actual RIA data, comparing theoretical and observed standard curves. The values were in fair agreement with each other, using polyclonal and monoclonal antibodies, and single-chain Fv fragments. As spreadsheet programs are popular in personal computers, they are useful for the purpose of characterizing antibodies and assessment of assay systems.

Introduction

Since the first development of radioimmunoassay (RIA) by Berson and Yalow[1], immunoassay has been one of the major techniques for determination of trace substances, especially in the field of biomedical and biochemical analysis. Today, non-radioactive methods based on enzyme-labeling such as enzyme-linked immunosorbent assay (ELISA), chemi- or bioluminescent enzyme immunoassays (CLEIA, BLEIA) are mainly employed in laboratory medicine. However, radioimmunoassay is still useful for fundamental ana-

lytical chemistry. In the process of developing new assay systems, we have to choose the most suitable antibody among those elicited in immunized animals. For screening, it is necessary to characterize each antibody using e.g. the affinity constant (Ka).

Recently, a variety of noncompetitive hapten immunoassays have been proposed[2,3]. These assay systems employ new-generation antibody reagents such as anti-idiotype or anti-metatype antibodies. Conventional anti-hapten antibodies often lack enough specificity and/or affinity mainly due to the limited B cell repertoire in immunized animals. Single-chain Fv fragments will be also useful to modify the properties of wild-type antibodies using antibody engineering. In these studies, characterization of antibodies for screening is important.

RIA has advantages for the purposes mentioned above, due to the structure of the labeled antigen. Immunoreactivity is therefore almost the same as that of the non-labeled antigen. In addition, we can easily know the amount of these antigens from the measured radioactivity, helpful for stoichiometric analysis.

Spreadsheet programs are useful for processing large quantities of data routinely and automatically, as well as drawing graphs with calculated values and regression analysis. Spreadsheets are also very popular software for personal computer users. In this study, we have prepared worksheet templates for constructing theoretical standard curves in competitive RIA, and calculating the Ka value, to characterize antibodies and assess assay systems. To evaluate the utility of these worksheets, theoretically simulated curves were compared with obtained RIA data.

Apparatus and Methods

Software: Excel® (Microsoft Corp., Redmond, WA) was used as the spreadsheet program for simulation. Employed versions were 2002 on Windows XP, X on Mac OS X, 98 and 2001 on Mac OS 9.x, respectively depending on personal computers used.

Antibodies: Anti-hapten antibodies from 4 categories used in these experiments; polyclonal anti-estriol 3-sulfate 16-glucuronide antiserum[4], monoclonal anti-cortisol antibody[5], single-chain Fv fragment (scFv) from an anti-11-deoxycortisol antibody[6], anti-11-deoxycortisol scFv-alkaline phosphatase (ALP) fusion protein[7] have been prepared previously in our laboratories.

RIA: RIA was carried out according to the previous reports[4-6]. The [^{3}H]-labeled antigen (330 Bq; 500 μl) and a series of standard antigen (100 μl), each dissolved in 50 mM sodium phosphate buffer (pH 7.3) containing 0.9% NaCl and 0.1% gelatin (GPB), were mixed with the corresponding antibody or scFv (diluted with GPB to bind 50% of the added tracer) (100 μl) and incubated overnight at 4°C. The bound (B) and free (F) fractions were separated by the dextran (0.05%)-charcoal (0.5%) method, and the bound radioactivity was counted with a scintillation cocktail (10 ml).

Scatchard plot: According to the method by Scatchard[8], Ka can be calculated by plotting the B/F ratio against B concentration as the negative value of the slope of the regression line. A template of the Excel worksheet was prepared with the following parameters: molecular weight [g/mol], specific activity [GBq/mmol] and added amount of the labeled antigen [dpm]. By entering the variables, added amount of non-labeled antigen [pg] and

corresponding bound radioactivities observed [dpm], the worksheet calculates B [pM] and B/F, then plots the graph and shows the equation by linear regression to get Ka.

Simulation of competitive RIA: In a competitive immunoassay, the B/B_0 value can be expressed as a function of the added non-labeled antigen as the following equation[9]:

$$B/B_0=\frac{A}{X+A}\times \frac{Kd+X+A+R-\sqrt{(Kd+X+A+R)^2-4R(X+A)}}{Kd+A+R-\sqrt{(Kd+A+R)^2-4RA}} \quad (1)$$

where A, X, R are the concentration of the labeled antigen, non-labeled antigen, and antibody in the reacting mixture, respectively. Kd is the dissociation constant (equal to the reciprocal of Ka) between the antibody and the antigen. When the assay is performed with R that the antibody can be bind with the added labeled antigen at the rate of p, R can be expressed with Kd, A and p:

$$R=(p/(1-p))Kd+pA \quad (2)$$

Thus, $R=Kd+A/2$ is obtained in the assay condition described above.

Based on these equations, a worksheet template was prepared with following parameters: molecular weight of the analyte hapten [g/mol], specific activity [GBq/mmol] and added amount of the labeled antigen [dpm], Kd [pM], and the volume of the reacting mixture [ml]. If the B_0 value is far from 50% of the added tracer, the binding rate p will be additionally used as in equation (2). By entering the added amount of non-labeled antigen [pg], the theoretical value of B/B_0 will be calculated and a graph for the standard curve will be also plotted.

Results and Discussion

Initially, we have prepared the worksheet for the Scatchard plot to get the Ka value, one of the characteristics of antibodies, and an essential parameter for the theoretical curve. The concentration of the bound antigen and the B/F ratio should be calculated from the data. In practice, the experiment will be carried out as competitive RIA. Thus the worksheet requires a set of variables, radioactivity and weight of antigen, with parameters of molecular weight, specific radioactivity and assay volume. As a graph is simultaneously drawn with calculated values, the user can choose points for the plot to try for the most suitable Ka value (Fig. 1). The quality of fit can be checked by RIA simulation.

Our main efforts were focused on the other worksheet, constructing the theoretical standard curve in competitive RIA. As shown in equation (1), the B/B_0 value is a function of the concentration of the non-labeled antigen. However, in hapten immunoassays, this ratio is often plotted against the weight of antigen. Thus the worksheet was designed to accept entering the variables, the amounts of non-labeled antigen in weight units [pg], and the labeled antigen in units of radioactivity [dpm]. They are converted to concentrations using the parameters of molecular weight, specific activity, and assay volume. It is convenient to use the obtained data directly, while the user can also start the calculation from concentrations.

The evaluation of these two worksheets was carried out by applying them to actual RIA data. Considering the possibility that there could be some difference in reaction system with the kind of antibody, anti-hapten antibodies from 4 categories were employed in the experiment; 1) polyclonal antibody (antise-

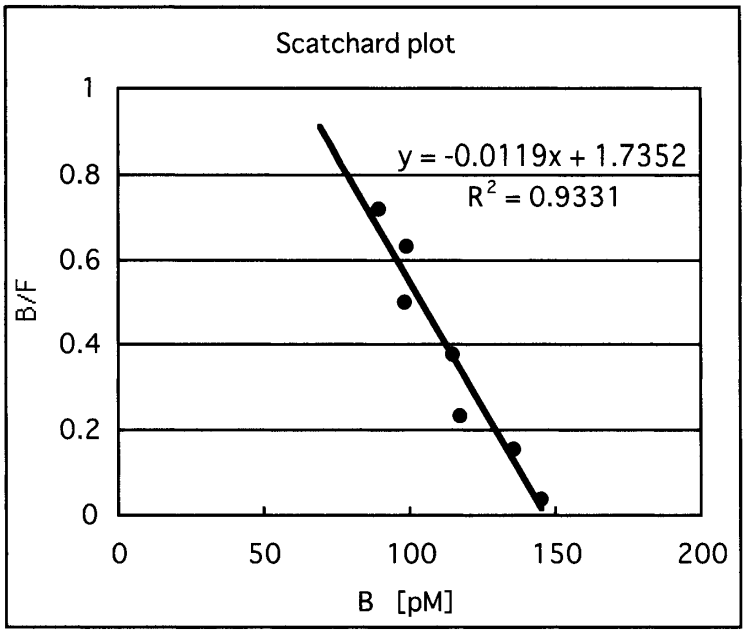

Figure 1. Scatchard plot on the worksheet.

rum), 2) monoclonal antibody, 3) single-chain Fv fragment (scFv) from a monoclonal antibody, and 4) scFv-enzyme fusion protein. ScFv is a polypeptide where VH and VL domains from an immunoglobulin are linked with the insertion of flexible linker peptide using antibody engineering, and it is possible to introduce mutations into the variable region of the wild-type antibody. The ScFv-enzyme fusion protein is a new type of a marker molecule in which a scFv is linked with an enzyme 1 : 1 by combination of the genes corresponding to both peptides, and is also available for stoichiometric processing of immunochemical reactions. The worksheets will be useful to assess the characteristics of these binding proteins changed from the wild-type antibody.

First, Ka values were calculated in these antibodies to use for the construction of theoretical curves. The values were in the order of $10^8-10^{10}\,M^{-1}$, comparable to anti-steroid antibodies previously reported. Ka values of scFv and scFv-ALP, prepared from the same monoclonal antibody, were estimated to be 1.19×10^{10} and $1.06\times10^{10}\,M^{-1}$, respectively. The Ka of the original antibody had been estimated to be $2\times10^{10}\,M^{-1}$ [10]. This result shows that preparation of scFv from an immunoglobulin, or attachment of an enzyme to scFv had no effect on Ka in these cases. This is a practical usage of the worksheet.

Then, theoretical and observed curves were compared. They were in fair agreement in the case of polyclonal antibody and scFv, suggesting that these worksheets are working well. In the case of a monoclonal antibody, a distortion was observed in the range up to 200 pg. This may be due to an experimental error because of the comparatively low Ka value resulting in decreased sensitivity (Fig. 2). In RIA with scFv-ALP, the theoretical curve did not agree with the data, although the Kd value was calculated by the observed assay data. We tried to change several parameters used in this simulation to fit the theoretical curve with the observed, and found a good agreement when the

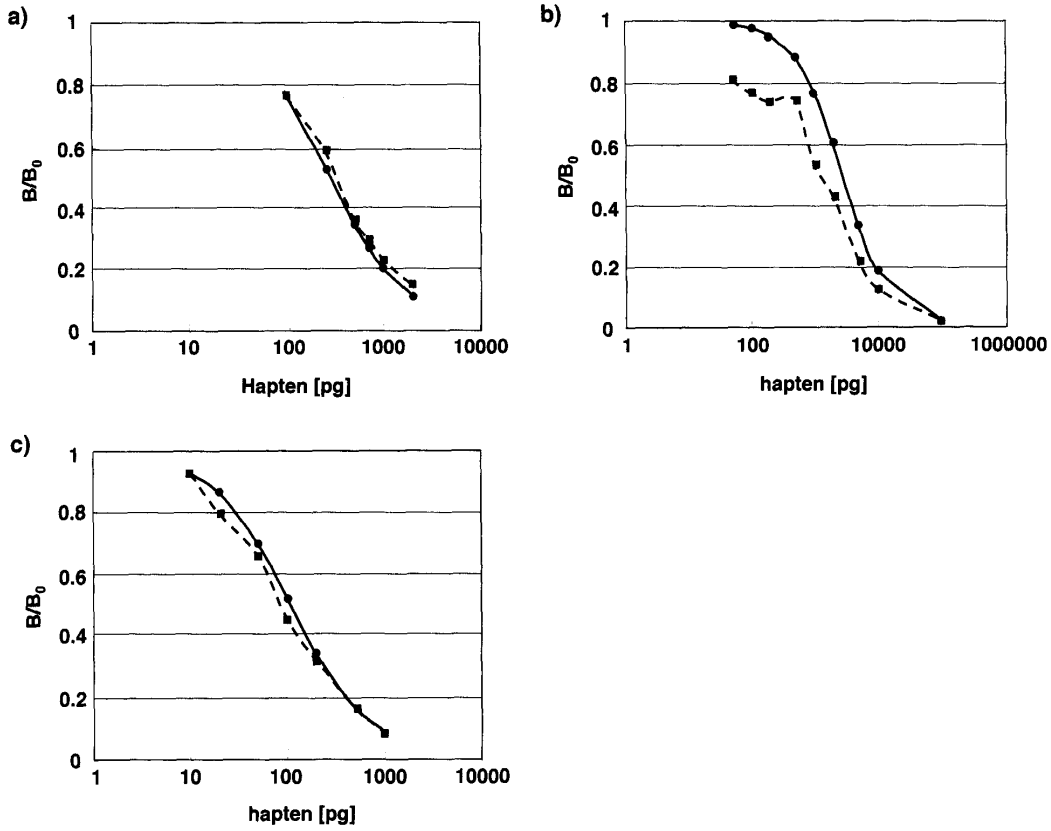

Figure 2. Comparison of theoretical and observed standard curves in competitive RIAs. a) polyclonal anti-estriol 3-sulfate 16-glucuronide antiserum ($Ka=2.03\times10^{9}\ M^{-1}$), b) monoclonal anti-cortisol antibody ($Ka=2.53\times10^{8}\ M^{-1}$), c) scFv from anti-11-deoxycortisol antibody ($Ka=1.19\times10^{10}\ M^{-1}$).
—— theoretical curve, - - - observed curve

Kd value became 1/10 (Fig. 3). We can not explain this difference using only the static parameter Kd, and may have to consider the dynamic parameter, both association and dissociation rate constants[11]. This case shows that we can find a possibility of some trouble in the assay system using these worksheets.

Another usage of the worksheet is that we can estimate the theoretical detection limit in competitive RIA for comparison, to evaluate the developing noncompetitive hapten immunoassays. We have developed a single-antibody immunoenzymometric assay of 11-deoxycortisol using ScFv-ALP fusion protein mentioned above[7], and the detection limit has been estimated to be 20 amol (7 fg)/assay. By using this worksheet recursively, the limit in competitive RIA was calculated to be 100 fmol/assay ($B/B_0=0.95$), showing that the noncompetitive method is five thousand times more sensitive.

Conclusion

Based on the equilibrium theory, we have prepared worksheets for simulation of competitive RIA and Scatchad plots. The utility of these worksheets was confirmed by comparing theoretical and observed standard curves. The spreadsheet program can process a large quantity of data routinely and automatically. In

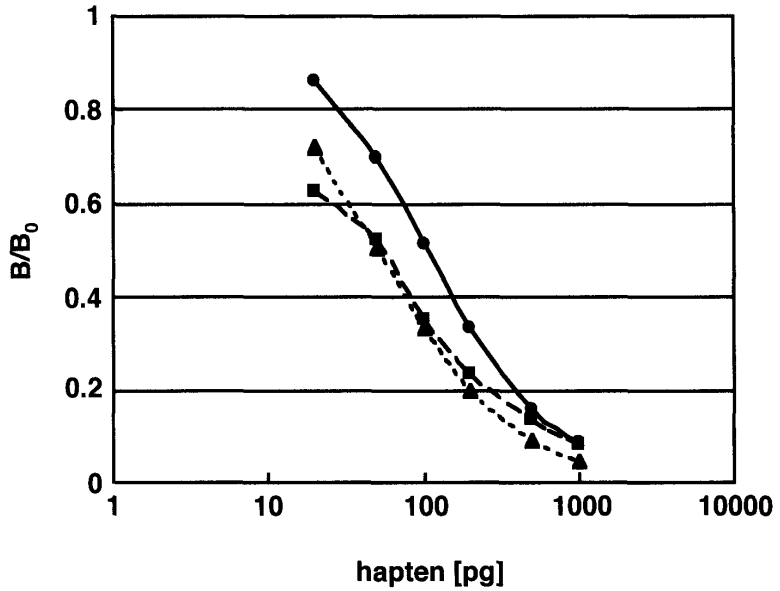

Figure 3. Comparison of theoretical and observed standard curves in RIA using anti-11-deoxycortisol scFv-ALP.
—— theoretical curve with Kd (94.3 pM) estimated from actual RIA, - - - observed curve, ····· simulated curve with Kd 1/10 of observed value.

addition, it is very popular and often is preinstalled in personal computers. These worksheets will be useful to characterize antibodies for screening and to evaluate assay systems.

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