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Measurement of the Affinity Constant of Monoclonal Antibody to Human Apolipoprotein A-I by ELISA

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Abstract: The present study was undertaken to determine the dissociation constant (Kd) of monoclonal antibody to human apolipoprotein A-I (apo A-I) using enzyme-linked immunosorbent assay (ELISA). First the monoclonal antibody was incubated in solution with the antigen until the equilibrium was reached; then the free antibody which remains unsaturated at equilibrium was captured by binding to antigen on the microtiter plate and be measured by a classical indirect ELISA. The value of Kd determined from Scatchard plot was 0.625×10^{-9} for purified antibody and 0.720×10^{-9} for unpurified antibody. This method was valuable for the measurement of true dissociation constant and found to be simple, reproducible, and accurate.

Key Words: Affinity constant, ELISA, Monoclonal antibody.

INTRODUCTION

The assay system determining the affinity constants for antibody-ligand complexes has been extensively investigated. Many methods for determining such binding constants have been published; for instance, ELISA,[1,2,4,7,8,10,13,15] radioimmunoassay(RIA),[5,11,12] fluorescein immunoassay[3] and surface plasmon resonance[9,18].

Many of these methods rely on binding to a molecule immobilized on a solid phase. This can be a problem: one is generally interested in the binding interactions in the solution phase, and these interactions can be modified substantially on the solid phase due to, for example, steric effect, multivalency and denaturation[14]. It is best to establish binding equilibrium in the solution phase. If solid-phase binding is required for separation of bound and free species, it should do so without perturbing the solution-phase equilibrium. In this work, we describe a simple, general method for the determination of the dissocia-tion constant using the ELISA system.

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MATERIALS AND METHODS

1. Preparation of apo A-I

Apo A-I was prepared according to the method of Yoon et al[17]. Pooled human sera were freshly obtained from Jeil Hospital (Seoul). 1 mM phenylmethyl sulfonyl fluoride and 1 mM aprotinin and 0.02% sodium azide were added and they were included in all solutions during the isolation procedure. HDL was isolated by sequential centrifugation method at density range of 1.08-1.21 g/ml. HDL was dialyzed extensively against 0.9% NaCl and 0.01% EDTA solution (pH 7.4). Apo A-I was prepared by delipidating the

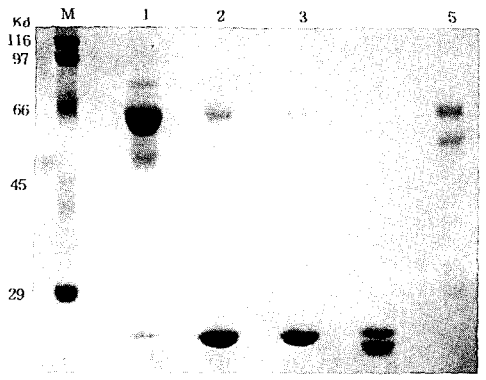

Figure 1. 10% SDS-PAGE of purified apo A-I. M, protein molecular weight marker; lane 1, human serun; lane 2, delipidated HDL; lane 3, apo A-I of our preparation; lane 4, a commercially obtained apo A-I (Sigma, A9284); lane 5, bovine serum albumin(BSA).

HDL with a mixture of ethanol and ether (v:v= 3:2) at 4°C and subsequently by gel chromatography on Sephacryl S-200 in 0.01 M Tris-HCl buffer (pH 8.0) containing 6 M urea, 0.02% NaN_3, 1mM sodium dodecyl sulfate (SDS) and 1mM EDTA. 50 mg of delipidated HDL-protein was applied to a 2.5×100 cm column and fractionated. Peak fractions were loaded onto 10% SDS-polyacrylamide gel for preparative electrophoresis and thus, tenacious albumin contamination from the apo A-I preparation was removed by electroelution. Purified apo A-I was dialyzed against 0.9% NaCl and 0.01% EDTA solution (pH 7.4) and stored at 4°C. The purity was confirmed by 10% SDS-PAGE (Fig. 1).

2. Production of monoclonal antibody to apo A-I

Female Balb/c mice were immunized initially by intraperitoneal injections with 20μg of apo A-I emulsified with an equal volume of Fruend's complete adjuvant plus dead *Salmonella* organism. The mice were boosted by intraperitoneal injection of the same amount of antigen emulsified in Freund's incomplete adjuvant after 14 days. Thereafter, the mice were boosted at 1-week interval with apo A-I in phosphate-buffered saline (PBS)

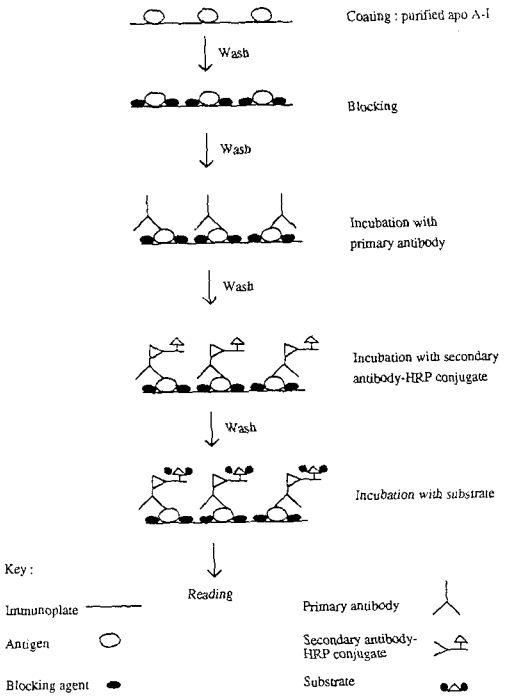

Figure 2. Schematic diagram of noncompetitive ELISA. This method is used to screen the positive clone of monoclonal antibodies and to determine the affinity constant of monoclonal antibody.

until test sera taken from the mice showed good titers against apo A-I in ELISA. Mice were then a final intravenous injection of apo A-I 3 days prior to the fusion.

The fusions were carried out according to the method of Galfre *et al*[6]. using 5×10^7 myeloma cells(Sp2/0-Ag-14) and $1\text{-}2 \times 10^8$ spleen cells. After fusion, cells were resuspended in RPMI 1640 (Sigma) containing 10% fetal bovine serum and final concentrations of 10 mM glucose, 20 mM sodium bicarbonate, 20 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol in addition, and distributed into flat-bottom 96-well culture plates (0.1 ml per well) over a mouse macrophage feeder layer (3×10^5 cells/well). The cultures were maintained in a humid incubator containing 5% CO_2. On the following day, 0.1 ml of a selective medium (HAT; 1×10^{-4}M hypoxanthine, 4×10^{-7}M aminopterin and 3×10^{-5}M

thymidine) of double strength was added to each well. Hybrid clones began to appear macroscopically 7-15 days after the fusion, supernatants were screened for the antibodies to apo A-I by an ELISA method (Fig. 2), and the positive clones were finally obtained by serial limiting dilutions.

3. Isotyping

The immunoglobulin class and subclass of each monoclonal antibody were assessed by both antigen-dependent and antigen independent ELISA using a commercialized kit (Pierce #37501). For antigendependent analysis, total lipoprotein-enriched mixture (LEM; 0.5μg in 100μl) was coated to microtiter plates, culture supernatant of monoclonal antibody for the determination was added to the wells, and ELISA was done using one of rabbit antisera raised against isotypes of mouse immunoglobulins as the second antibody. Horseradish peroxidase(HRP)-conjugated, goat anti-rabbit IgG(H+L) was used as the third antibody and absor-bances were read at 450nm. For antigen-independent analysis, goat anti-mouse Ig(G+A+M) was coated to microtiter plate and monoclonal antibody was added to the wells. ELISA was done using one of the rabbit antisera against isotypes of mouse immunoglobulins and HRP-conjugated, goat anti-rabbit Ig (H+L).

4. Affinity measurement using ELISA: Free-capture mode

The antigen at various concentrations was first incubated in solution with the antibody at constant concentration until equilibrium was reached. The concentration of free antibody was then determined by an indirect ELISA. The antigen at various concentrations (4×10^{-10}M to 2×10^{-7}M) was mixed with a constant amount of antibody (3×10^{-10}M) in 0.1 M potassium phosphate, 2 mM EDTA , pH 7.8, supplemented with 10mg/ml BSA. After overnight incubation at 4°C, 150μl of each mixture was transferred and incubated overnight 4°C into the wells of a microtiter plate previously coated with apo A-I (150μl per well, at 1μg/ml in 50 mM sodium carbonate, pH 9.6 overnight at 4°C), in which free antibody is captured by binding to antigen on the well. After washing with PBS supplemented with 0. 5% Tween 20, the bound immunoglobulins were detected by adding rabbit Ig with specificity against mouse IgG coupled with HRP and measuring the HRP activity retained in each well (Fig.3).

Dissociation constant was calculated by the modified Scatchard equation[16].

RESULTS

1. Production of monoclonal antibody

From the serial steps of hybridoma production, we finally selected and cloned a hybridoma producing anti-apo A-I monoclonal antibody of IgG type, which was designated as Mab A12. The heavy and light chain isotypes of antibody were determined by an antigen-dependent and antigen-independent ELISA and this analysis revealed that Mab A 12 had IgG2a heavy chain and κ light chain (Fig. 4). The binding specificity of Mab A12 was analyzed by immunoblotting analysis and reacted well with both isolated apo A-I and HDL in whole serum (not shown). Monocl-

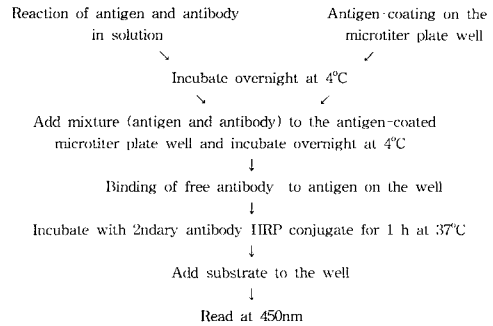

Figure 3. Schematic diagram for the method employed to determine the antibody binding constant, in which free antibody is captured by binding to antigen on the microtiter plate.

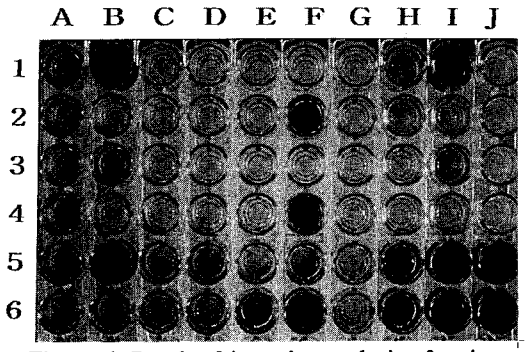

Figure 4. Result of isotyping analysis of anti-apo A-I monoclonal antibodies. In the antigen-dependent ELISA, total lipoprotein-enriched mixture (LEM; 0.5μg/100μl) was coated to microtiter plate, monoclonal antibody, Mab A12 was added to the wells, and proceeded for the 2nd antibody. HRP-conjugated goat anti-rabbit IgG (H+L) was used as the 3rd antibody. In the antigen-independent ELISA, same procedures were performed, but wells were coated with antibody working solution (goat anti-mouse Ig (G+M+A)). 1 and 2, LEM coated as antigen; 3 and 4, purified apo A-I coated as antigen; 5 and 6, antigen-independent assay; A, IgG1; B, IgG 2a; C, IgG2b; D, IgG3; E, IgM; F, IgA; G, light chain λ; H, light chain κ; I, normal rabbit serum; J, positive control (antigen-independent assay).

onal antibody Mab A12 used in this assay was affinity-purified on a protein A column.

2. Determination of affinity constant

The method - free capture mode employed to determine the antibody affinity constant is shown in Figure 3. In the free capture mode, the monoclonal antibody at a constant concentration is incubated long enough with various quantities of antigen in solution until equilibrium was reached. The amount of unbound antibody in the liquid phase could be bound to antigen on the microtiter plate wells and then be monitored by a classical indirect ELISA. The antibody at total concentration i_o is incubated with the antigen at a given concentration, the free antibody concentration, i, will be related to the absorbance A measured in the ELISA, by the following equation:

$$\frac{i}{i_o} = \frac{A}{A_o}$$

where A_o is the absorbance measured for the antibody in the absence of antigen. However, this will be true only if no readjustment of the equilibrium in the liquid phase occurs during the incubation of mixture in the coated wells. It was varified that this readjustment is negligible under the experimental conditions we used (i.e., quantity of antigen coated in the wells, and time of incubation of the mixtures in the wells). For the purpose, after incubating the antibody at various known concentrations in the coated wells during the appropriate time, the content of each well was transferred in to another coated well and incubated for the same time and the trapped antibody was then detected by ELISA.

The validity of first equation being established, we could easily calculate the concentrations x (of bound antibody) and a (of free antigen) at equilibrium from the mass conservation equations:

$$x = i_o - i \text{ and } a = a_o - x$$

where a_o is the total concentration of antigen, and i_o and i, respectively, the total and the free antibody site concentrations.

x, a and i_o are related to the dissociation constant Kd of the equilibrium by the Scatchard equation:

$$\frac{x}{a} = \frac{1}{Kd}(i_o - x)$$

From first equation, x and a are related to the absorbances measured in the ELISA:

$$x = i_o \times \frac{A_o - A}{A_o}$$

$$a = a_o - i_o \times \frac{A_o - A}{A_o}$$

Figure 5 shows the result in which v is the fraction of bound antibody $A(x/i_o)$ and a the concentration of free antigen at equilibrium. A straight line was obtained and Kd value obtained from the linear regression is 0.625×10^{-9}.

Furthermore, the ELISA method can also be

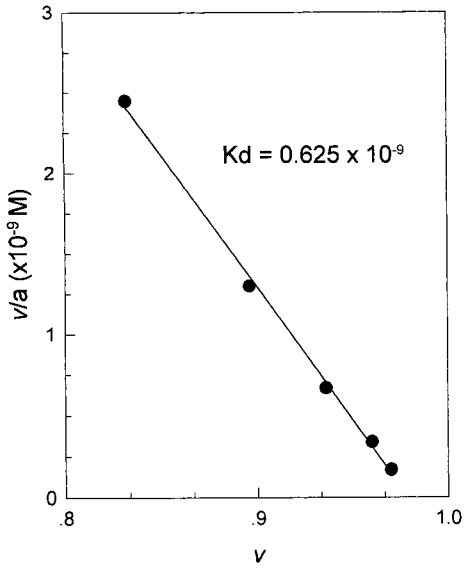

Figure 5. Scatchard plot of the binding of apo A-I to purified anti-apo A-I monoclonal antibody IgG 2a measured by the ELISA. υ is the fraction of the bound antibody and a the concentration of free antigen at equilibrium. υ corresponds to $(A_o\text{-}A)/(A_o)$.

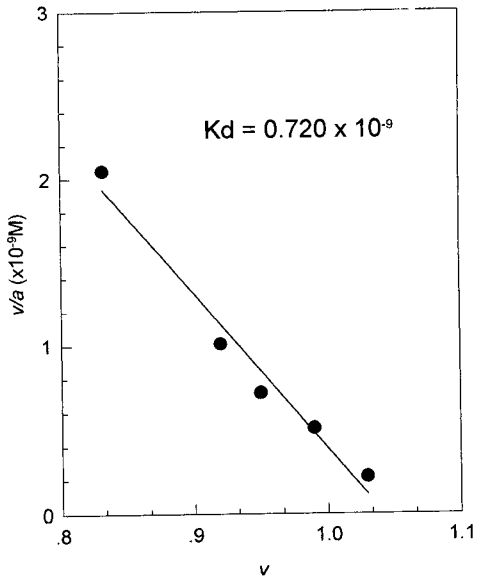

Figure 6. Scatchard plot of the binding of apo A-I to unpurified monoclonal antibody measured by the ELISA. Crude hybridoma culture supernatant was used as unpurified form.

applied to the determination of the affinity constant in solution, even in the case of unpurified antibodies (e.g. crude hybridoma culture supernatant). The results obtained from Scatchard plot are shown in Fig. 6. The Kd values deduced from the slope calculated from linear regression is 0.720×10^{-9}. The only requirement is that the total antigen concentration should be sufficiently higher than the antibody concentration.

DISCUSSION

The results reported demonstrate that the method proposed for studying the association-dissociation equilibrium between a monoclonal antibody and the corresponding antigen, based on the use of an ELISA to measure the concentration of free antibody at equilibrium, gives reliable values of the real dissociation (or affinity) constants of the system in solution [3,12]. The commonly used ELISA method measures directly the interaction of the antibody with the immobilized antigen (or vice-versa) and therefore does not permit the measurement of the true equilibrium dissociation constant since the antigen and the antibody are in separate phases.

We determined dissociation constant for monoclonal apo A-I antibody using the free-capture mode. This method is a particularly simple way to estimate Kd. A Scatchard plot of this result provides values of 0.625×10^{-9}M for purified antibody and 0.720×10^{-9} for unpurified antibody and this agreement indicates that the method is valuable for the measurement of the true dissociation constant. The values by our method are a little higher than the dissociation constant obtained by other researchers[3,4,7]. Indeed, this method offers a wide range of advantages over those commonly used to measure equilibrium constants. First, from a practical point of view, it is as easy to set up as a classical indirect ELISA. Therefore, it can be repeated on large series of experimental points and permits screening of variety of conditions such as temperature, buffer composition, antigen analogs.

Furthermore, only minutes amounts of reagents (antigen or antibody) are required to perform the measurements. Finally, the experimental results are easily interpretable in view of the very small dispersion and excellent reproducibility of the data[3,5].

In addition to these practical aspects, the ELISA method we propose for measuring affinities offers several theoretical advantages. This method deals with unmodified molecules in solution and needs no hazardous radiochemicals which are mainly used for the measurement of affinity constant. As discussed by Friguet *et al.* (1985) proteins may undergo denaturation when adsorbed to microtiter plates and thus conformational changes of the antigen occurring in solution would be hindered when the protein is immobilized in the solid phase.

The specificity of the interaction between the monoclonal antibody and the immobilized antigen on the one hand, and between the monoclonal antibody and the immunoenzymatic conjugate on the other hand, is such that the ELISA can easily be performed even if the monoclonal antibody is not purified. This allows one as shown in the present work, to obtain reliable values of equilibrium constants without needing to purify the antibody to homogeneity. It is not even necessary to titrate the antibody in the preparation provided the measurements at equilibrium are performed under conditions where the total antigen concentration is in large excess over the total antibody concentration. Because of the sensitivity and the specificity of the detection of free antibody by ELISA, this condition can be fulfilled in most cases. This permits a satisfactory estimate of the true dissociation constant of an antibodyantigen complex even with a crude preparation of a monoclonal antibody such as a hybridoma culture supernatant.

In view of the simplicity, the precision and the theoretical reliability of this ELISA method for the determination of equilibrium constants, it is likely to be of immediate interest for studying the affinity of monoclonal antibodies specific for any protein antigen. It could however also be transposed to monoclonal antibodies specific for a hapten, by using a protein-coupled hapten for the coating in the ELISA, and eventually to any associationdissociation equilibrium between 2 different proteins.

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= 국문초록 =

효소면역 분석법에 의한 아포지단백질 A-I 단일클론항체의 친화상수의 측정

목원대학교 생물학과

윤 미 정 · 이 현 희

본 연구에서는 효소면역진단법을 이용하여 사람의 아포지단백질 A-I에 대한 단일클론항체의 해리상수 (Kd)를 측정하고자 하였다. 먼저 단일클론항체와 항원을 평형에 도달할 때까지 액체상에서 반응시킨 후, 평형상태에서 항원과 결합하지 못하고 남아있는 항체를 미세적정판에 결합되어 있는 항원과 반응시킨다. 그 다음 결합된 항체의 양이 효소면역분석법에 의하여 측정한다. 본 실험방법을 이용하여 측정된 아포지단백질 A-I에 대한 단일클론항체의 해리상수는 정제된 형태의 경우 0.625×10^{-9}이었으며, 정제되지 않은 하이브리도마 배양액의 경우 0.720×10^{-9}이었다. 이 방법은 간단하고 재생성이 높으며, 정확하고 방사선 동위원소를 사용하지 않는 등 많은 장점을 가진 것으로 생각된다.

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