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Review

Affinity electrophoresis and its applications to studies of immune response[1]

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Abstract

Affinity electrophoresis (AEP) is a useful technique for separation of biomolecules such as plasma proteins, enzymes, nucleic acids, lectins, receptors, and extracellular matrix proteins by specific interactions with their ligands in electric fields and for the determination of dissociation constants for those interactions. Two-dimensional affinity electrophoresis (2-D AEP), which was newly developed by a combination of isoelectric focusing with AEP, has been used for studies on immune response to haptens. Antihapten antibodies, which were induced by immunization of a mouse with the hapten-conjugated bovine serum albumin, were separated by 2-D AEP into a large number of groups of IgG spots with a few microliters of antiserum. Each group of spots showed an identical affinity for the hapten but different isoelectric points as in the case of monoclonal antibodies specific to the hapten. This enabled us to study the diversification and affinity maturation of antihapten antibodies in the course of immunization of a single mouse. Furthermore, effects of a carrier and a hapten array on the production of antihapten antibodies and the cause of charge heterogeneity of monoclonal antibodies were also examined to understand the molecular basis of the immune response in vivo.

Keywords: Review; Immune response; Hapten; Antihapten antibodies

Contents

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[1]The authors recommend reviews (Ref. No.10 and 49) to readers for further information on applications of affinity electrophoresis to studies of other biospecific interactions such as enzymes with their substrates and inhibitors, lectins with carbohydrates, and nucleic acids with proteins.

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1. Introduction

The first application of an electrophoretic technique to studies of immune response was performed by Tiselius and Kabat [1]. Antibodies were separated in the γ-fraction of human sera by free electrophoresis called Tiselius electrophoresis as shown in Fig. 1. In 1959, Nakamura et al. [2–6] developed a novel technique of electrophoresis called 'cross electrophoresis' which explored biospecific interactions such as enzymes with their substrates and inhibitors, antibodies with antigens, nucleic acids with protamines and modified nucleotides, and lectins with glycoproteins [2–6]. Techniques of rocket immunoelectrophoresis [7] and crossed immunoelectrophoresis [8] are both based on the same principles of cross electrophoresis. In 1972, Takeo and Nakamura reported the determination of dissociation constants of interactions between glycogen phosphorylases and glycogen by polyacrylamide gel electrophoresis [9]. The mobility of the enzyme was decreased by the specific interaction with glycogen which was entrapped in a meshwork of polyacrylamide gel, and the mobility change was proportional to the concentration of glycogen. The apparent dissociation constant (K_d) for the interaction was calculated from the mobility change as a function of glycogen concentration. This technique was later called 'affinity electrophoresis' (AEP) which was first proposed by Bøg-Hansen [11], Horějši and Kocourek [12] and Caron et al. [13]. Horějši and coworkers [14–16] and Nakamura et al. [17–19] developed the methods of immobilization of small molecular ligands on gel matrices for determining K_d values for interactions of lectins with saccharide derivatives and of enzymes with hydrophobic ligands and nucleotide cofactors such as nicotinamide adenine dinucleotides. AEP was first applied to analyses on immunoreactions by Takeo and Kabat [20]. They calculated K_d values for the interactions of dextran specific IgG myeloma proteins with dextran and isomaltose oligosaccharides. Recently, we developed a new technique of two-dimensional AEP (2-D AEP) which was used for the separation of antihapten antibodies in antisera. The antibodies were first separated by isoelectric focusing in the first dimension and then were separated by AEP in the second dimension. The technique of 2-D AEP enabled us to separate polyclonal antibodies into monoclonal antibodies. The term 'affinity electrophoresis' may now include all of electrophoresis techniques which explore biospecific interactions, and the techniques have been widely used for biomedical research and clinical chemistry [10].

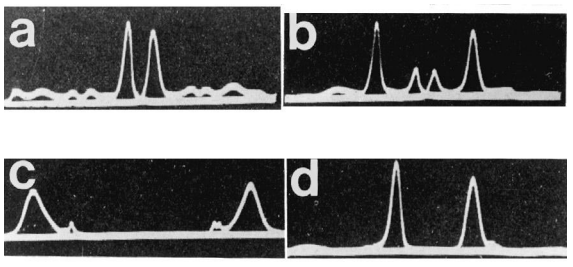

Fig. 1. Schlieren patterns of human serum proteins separated by Tiselius electrophoresis. The buffer system was 0.1 *M* veronal–HCl (pH 8.6). (after K. Shimao, in Japanese Electrophoresis Society Ed. Denkieidou-jikkenhou, Bunkoudou, Tokyo, 1976, pp. 38). (a) Normal serum, (b) myeloma serum, (c) γ-globulin, (d) myeloma protein.

2. Principles of affinity electrophoresis

2.1. Cross electrophoresis

Cross electrophoresis is a useful technique for separation of biomolecules by their specific interactions with their ligands in electric fields. The charged molecules interact with each other during migrating in support media without a molecular sieving effect, such as paper [4–6] and agarose gel [8]. The interactions result in torsion of electrophoretic patterns of the molecules specifically and in precipitation of the complexes of the molecules as in the case of antigen–antibody interactions (Fig. 2). This technique is quite powerful for separation of small amounts of biomolecules and analyses of biospecific interactions. However, it is difficult to determine kinetic parameters of the interactions because we cannot fix the concentrations of the ligands in the electric fields precisely.

2.2. Affinity electrophoresis

The kinetic parameters of biospecific interactions are determined by fixing the concentrations of lig-

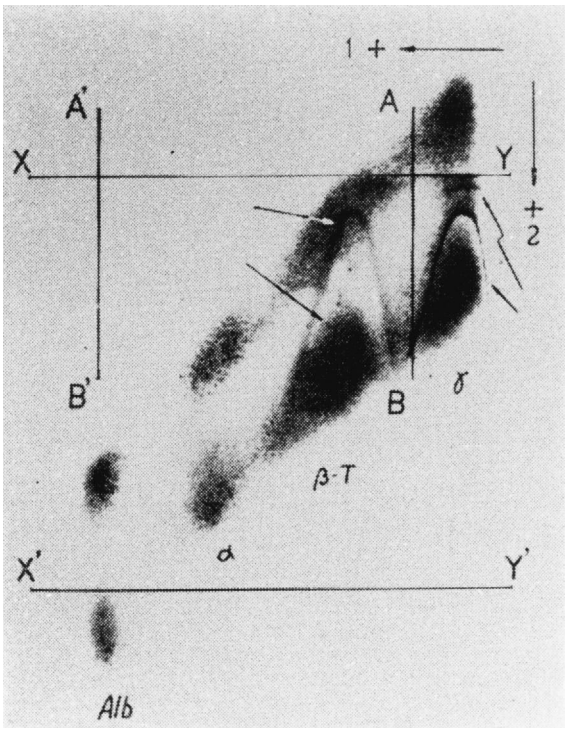

Fig. 2. Two-dimensional cross electrophoresis of antidiphtheria antiserum and diphtheria toxoid [4]. The antiserum was applied onto the line AB, and was electrophoresed to the line A′B′. Then, the toxoid solution was applied onto the line XY, and electrophoresed to the line X′Y′. The immunoprecipitin lines were formed as indicated by arrows in β- and γ-fractions of the antiserum.

ands in support media during electrophoresis, for instance, (1) trapping the macromolecular ligands in the gel matrices by molecular sieving effect such as polyacrylamide gel [9,22–24], (2) immobilizing the low-molecular-mass ligands by covalently attaching to the matrices [12–19], (3) adding the charged ligands to all through the continuous buffer systems for electrophoresis [25,26]. The dissociation constant (K_d) is defined as the value for the ligand concentration at which the specific biomolecule migrates half of the distance compared with the distance in the absence of the ligand [9]. There was a proposal for calculating the true K_d using the term of effective ligand concentration [15], however, the apparent K_d may be the most reliable value because of several practical limitations [27]. The K_d is calculated according to the affinity equation which was derived from the interaction between phosphorylase, P, and its ligand, glycogen, G, as expressed by the following equations [9].

$$P + G = PG \quad (1)$$

$$K_d = [P][G]/[PG] \quad (2)$$

When P migrates in a polyacrylamide gel trapping macromolecular ligand, G at a concentration, *c*, with disc electrophoresis system, a part of P combines reversibly with G to form the complex of PG,

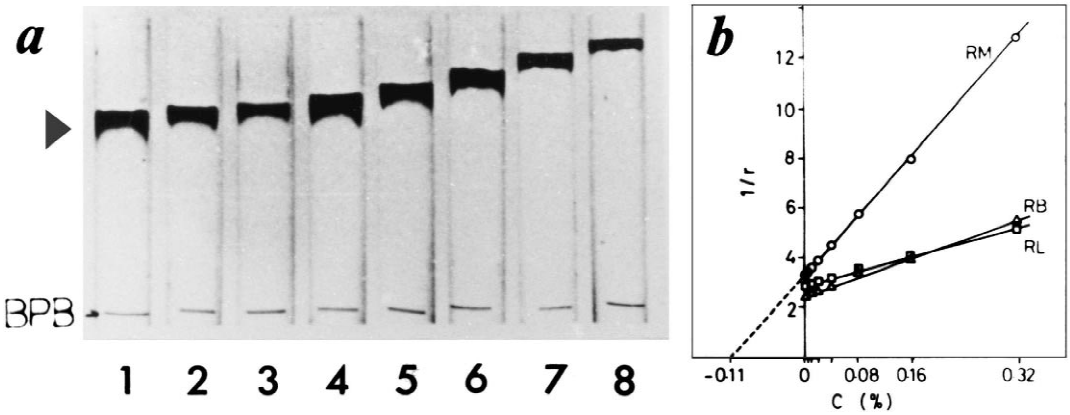

Fig. 3. Affinity plots for the interactions between phosphorylase and glycogen [9]. The band of phosphorylase (closed triangle) decreased its mobility according to the increase of the concentration (0 from 0.32%) of glycogen in the gels (panel a). The affinity plots, taking the reciprocal values of relative migration distance of the band ($1/r$) as the ordinate and the concentrations of glycogen (c) as the abscissa give a straight line, and its intercept of abscissa gives a negative value of dissociation constant (K_d) for the interaction between phosphorylase and glycogen (panel b).

resulting in a decrease of mobility of the P band. The extent of the decrease in mobility depends on c. The mobility of the P band becomes zero when all of P forms the complex of PG. Relative migration distance of the P band in the absence of G in the gel is expressed as R_0 and that of the P band at a concentration of c as r, and the following equation can be derived:

$$R_0/r = [P]_t/[P] \quad (3)$$

where $[P]_t$ is the total concentration of P, and $[P]_t = [P] + [PG]$. Eq. (3) is transformed to:

$$R_0/r = 1 + [PG]/[P] \quad (4)$$

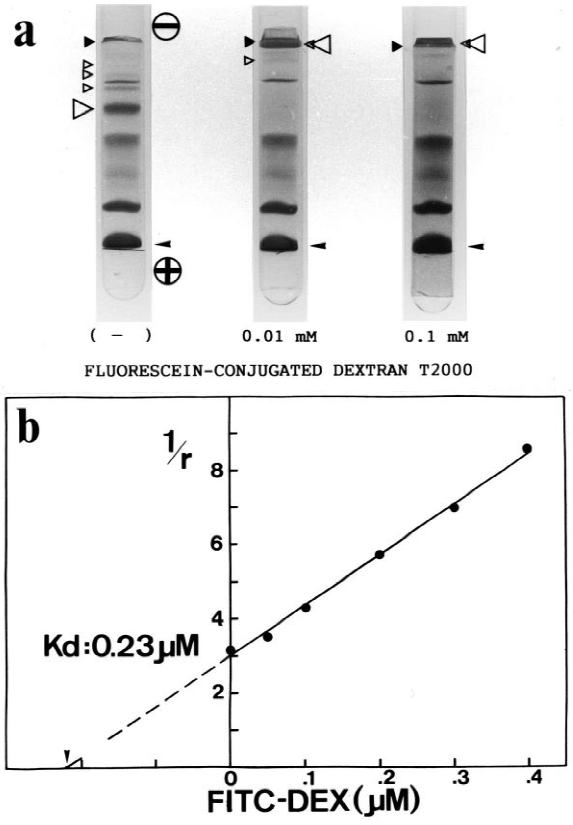

Fig. 4. Determination of dissociation constant (K_d) for the interaction between monoclonal antibody, FLD6 (IgG1 κ) and fluorescein isothiocyanate-conjugated dextran T2000 (FITC–DEX) by affinity electrophoresis. The band of FLD6 secreted from hybridoma cells in murine ascites fluids markedly decreased its mobility (large open triangles) according to the increase in the concentration of FITC–DEX (panel a). The affinity plots of $1/r$ of FLD6 against the concentrations of FITC–DEX give a straight line which intercepts the abscissa at a negative K_d value (panel b).

When Eq. (2) is substituted in Eq. (4), Eq. (5) is obtained:

$$R_0/r = 1 + [G]/K_d \quad (5)$$

[G] is the free G concentration in the gel. Under conditions where c is much higher than $[P]_t$, [G] is practically equal to c. Eq. (5) can be transformed to:

$$1/r = 1/R_0(1 + C/K_d) \quad (6)$$

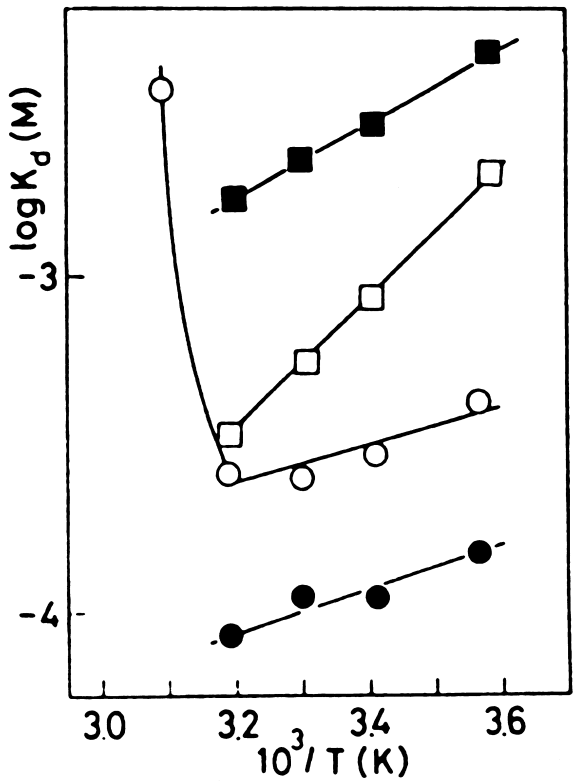

Fig. 5. Effects of temperature on the interactions between a monoclonal antibody, MOPC 315 (IgA λ) and 2,4-dinitrophenol (DNP)- and 2,4,6-trinitrophenol (TNP)-conjugated polyacrylamide [27]. The van't Hoff plots of logarithmic values of K_d for the interactions of MOPC 315 with DNP and TNP against the reciprocal values of absolute temperature gave straight lines. The thermodynamic parameters of the interactions could be calculated. The line (■) is for the interaction between MOPC 315 monomer and DNP, and the thermodynamic parameters $\Delta G = -3.4$ kcal/mol, $\Delta H = 5.2$ kcal/mol, and $\Delta S = 29$ cal/mol/deg. Other lines (□), (●) and (○) are for MOPC 315 dimer and DNP, MOPC 315 monomer and TNP, and MOPC 315 Fab′ and TNP, respectively. The plot for MOPC 315 Fab′ deviated from the line at 50°C and the increase in K_d indicated that the Fab′ markedly decreased its affinity for TNP.

As shown in Fig. 3, a straight line is obtained when Eq. (6) is plotted, taking $1/r$ as the ordinate and c as the abscissa. Its intercept with the abscissa gives a negative value of K_d. The principles of AEP have been described in more detail in the review by Takeo [10]. Fig. 4 shows the determination of K_d for the interaction between fluorescein isothiocyanate (FITC)-specific monoclonal antibody of FLD6 and its ligand of FITC immobilized on dextran which is trapped in the gel. When the antibody has a high affinity for the hapten ($K_d < \mu M$), it is needed to detect the antibody band of subnanograms in the gel. In such cases the bands of biomolecules are detected by highly sensitive detection methods using ELISA with chemiluminescence because of the limitation of the condition for calculating K_d, where the concentration of FITC ligand, c is much higher than that of the antibody.

3. Applications of affinity electrophoresis

3.1. 1. Determination of K_d values for immunoreactions

The first application of AEP for determining K_d values for immunoreactions was carried out by Takeo and Kabat [20]. They calculated K_d values for interactions between dextran specific murine myeloma proteins and dextrans, and analyzed the feature of the interactions with isomaltose oligosaccharides as inhibitors. Using the same procedure, K_d values for interactions of fructan specific myeloma proteins with oligosaccharides derivatives were obtained [28–35]. Tanaka et al. applied AEP to determine K_d values for the interactions between 2,4-dinitrophenyl (DNP) specific murine myeloma proteins, MOPC-315 and DNP derivatives [27]. They examined the effect of temperature on K_d values, and thermodynamic parameters calculated from the Van't Hoff plots for the interactions indicated that a light chain dimer with a low affinity for DNP derivatives might be formed at 50°C and the interactions were endothermic and hydrophobic as shown in Fig. 5. Conversely, the thermodynamic parameters for other biospecific interactions of concanavalin A with carbohydrates [36,37] and fibronectin with gelatin [38] indicated that those interactions were exothermic and hydrophilic. Mimura et al. [39] examined the effect of pH on K_d for an interaction between dextran-specific monoclonal antibody, 35.8.2H and dextran B512 (Fig. 6) by AEP named affinity titration [40] by which biomolecules were separated by their specific ligands in gels having pH gradients. The K_d

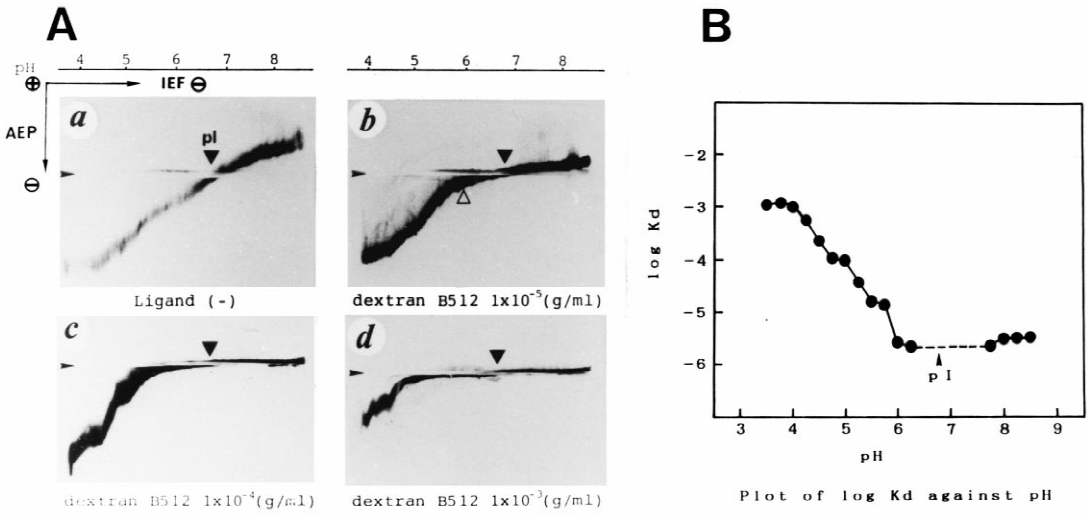

Fig. 6. Effects of pH on the interactions of a monoclonal antibody, 35.8.2H (IgG1 κ) with dextran B512 [39]. Affinity titration curves at different concentrations of dextran B512 are shown in panel A. The plots of K_d values at different pH values show that the affinity of 35.8.2H decreases its affinity for the dextran by lowering pH (panel B).

values became larger at lower pH, and the affinity of 35.8.2H for dextran markedly decreases under acidic conditions. Conversely, K_d values for the interactions between FITC-specific monoclonal antibody, FLD6 were not strongly affected by altering the pH (Nakamura et al., unpublished data).

3.2. Studies on immune response in vivo by two-dimensional affinity electrophoresis

The antibodies specific for a single epitope or hapten in antisera against a certain antigen or a hapten-conjugated carrier protein are so heterogeneous that a satisfactory resolution of the antibodies has still not been obtained by standard two-dimensional electrophoresis [41,42]. Takeo and coworkers [21,43] developed two-dimensional affinity electrophoresis (2-D AEP) by a combination of isoelectric focusing with AEP. Fig. 7 shows the patterns of affinity purified rabbit anti-DNP antibodies separated by 2-D AEP into a large number of groups of IgG spots interacted with DNP immobilized in gel matrices. Each of the groups of IgG spots showed an identical affinity for DNP but different isoelectric points (p*I*) [44–46]. Nakamura and coworkers

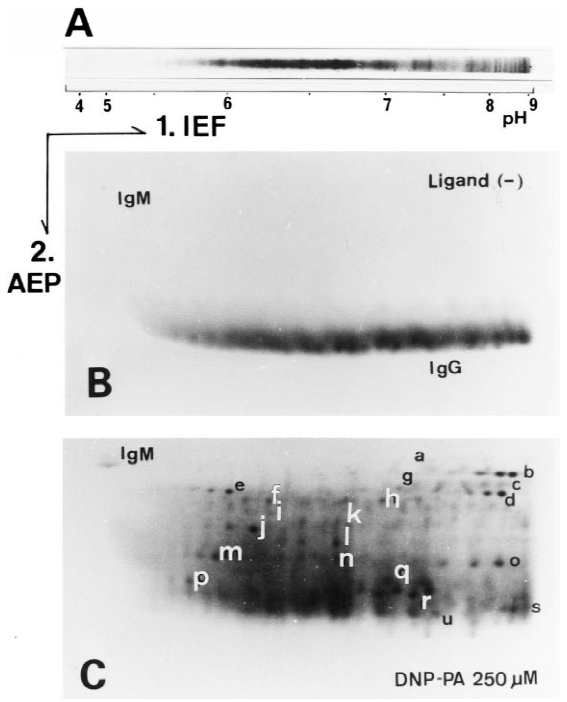

Fig. 7. Two-dimensional affinity electrophoresis (2-D AEP) of anti-DNP antibodies which were purified from rabbit antisera [44]. The antibodies were separated by the difference in their surface charges using isoelectric focusing in the first dimension (panel A), and then they were separated by the difference in their affinities for DNP-conjugated polyacrylamide using AEP in the second dimension in the absence of DNP (panel B) and the presence of DNP (panel C). The antibodies were finally separated into a large number of groups of spots (a to u) having identical affinities but different p*I* values.

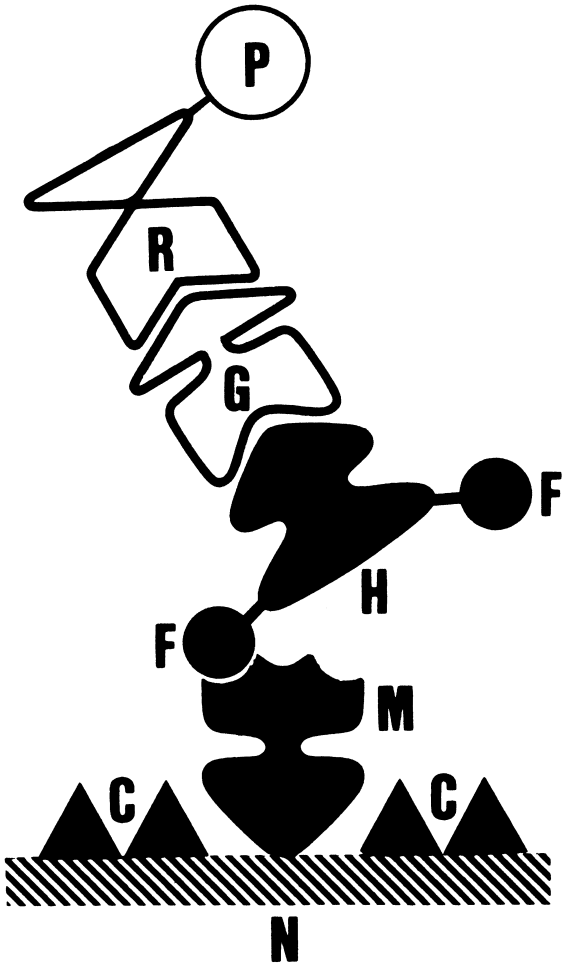

Fig. 8. Principles of staining of anti-FITC antibodies in antisera separated by 2-D AEP [48]. After 2-D AEP the antibodies are transferred to a nitrocellulose membrane and stained by ELISA method. The symbols indicate C, casein in skimmed milk; F, fluorescein thiocarbamoyl group; G, goat antihuman IgG antibody; H, human IgG; M, murine anti-FITC antibodies; N, nitrocellulose membrane; P, peroxidase (horse radish); R, rabbit anti-goat IgG antibody.

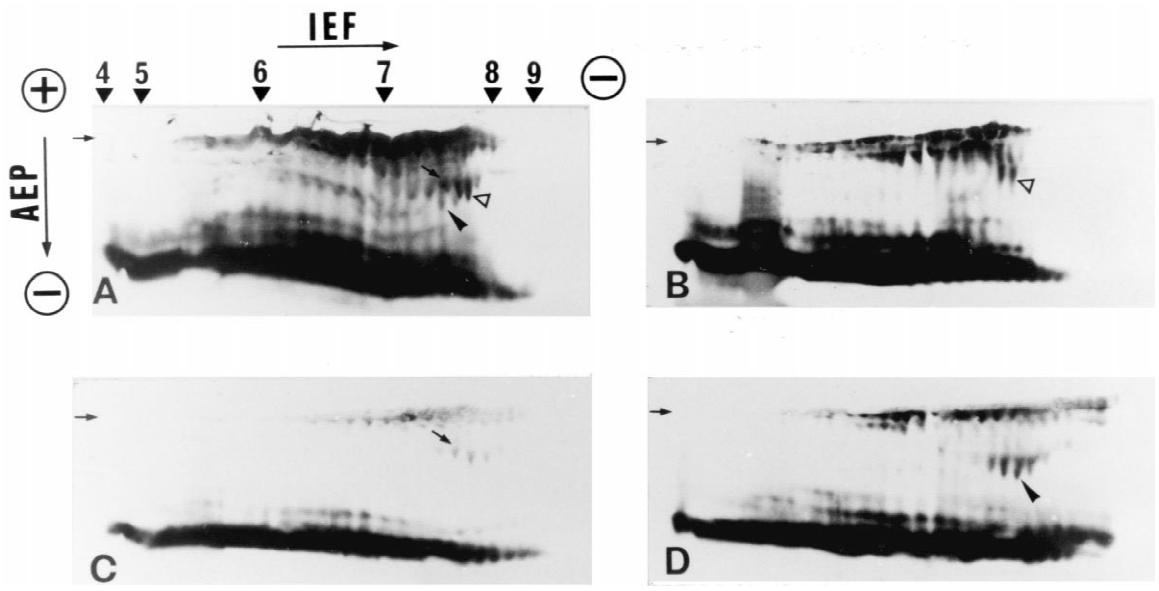

Fig. 9. 2-D AEP of IgG subclass of anti-FITC antibodies in antiserum obtained from a single mouse which was immunized with FITC-conjugated serum albumin [47]. The subclass specific IgG spots were stained by ELISA method using antibodies specific for murine IgG subclass, IgG1 (panel B), IgG2a (panel C), and IgG3 (panel D). Those spots were stained by the method using anti-murine IgG Fc γ antibodies (panel A) as indicated by the symbols of open triangle for IgG1, arrow for IgG 2a, and arrowhead for IgG3.

[47,48] separated anti-DNP and anti-FITC antibodies by 2-D AEP with 4 μl of murine antisera against DNP- and FITC-conjugated serum albumin without affinity purification. The principles of the specific staining of the antihapten antibodies are shown in Fig. 8. We confirmed that the hapten specific antibodies could be separated into a large number of groups of IgG spots and each group of the IgG spots having an identical affinity for the hapten but different p*I* was in a certain IgG subclass (Fig. 9). Furthermore, IgG1 κ monoclonal antibodies specific to FITC and DNP from hybridoma cell clones of FLD6 and 1B7-11 were separated by 2-D AEP into several spots having an identical affinity for the hapten but different p*I* values (Fig. 10). We concluded that each of the groups of IgG spots was derived from a single clone of B cells specific to the hapten [48,49]. This enabled us to analyze the diversification and affinity maturation of the antibodies in vivo using a single mouse in the course of immunization with the hapten-conjugated serum albumin (Fig. 11). Wang et al., [50] examined the effects of antigen doses on the production of anti-FITC antibodies in a single mouse and the time course of IgG subclass switch of the antibodies. We showed that the delay of the production of IgG2a in

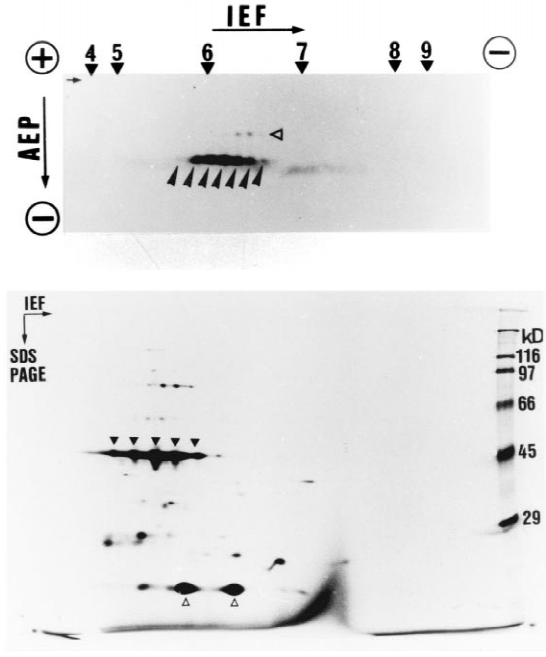

Fig. 10. Molecular heterogeneity of a monoclonal antibody, FLD6, separated by 2-D AEP [48]. The monoclonal antibody was separated into several spots having an identical affinity to FITC–DEX but different p*I* values as indicated by arrowheads in upper panel, and the charge heterogeneities in the heavy chain and the light chain were shown by two-dimensional electrophoresis as indicated by closed triangles and open triangles in the lower panel.

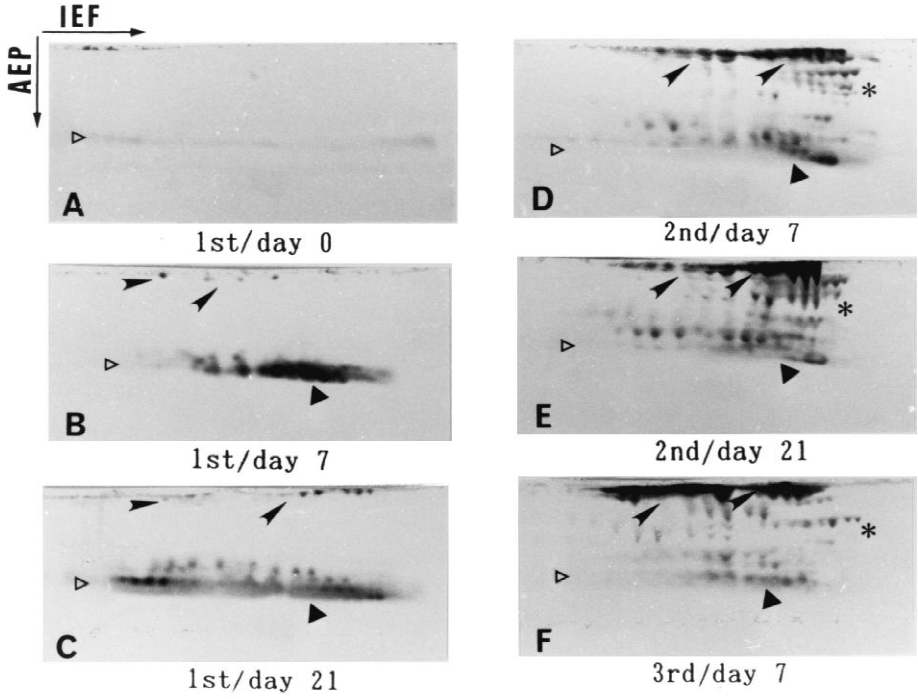

Fig. 11. Antigen induced diversification and affinity maturation of anti-FITC antibodies in a single mouse analyzed by 2-D AEP [50]. The BALB/c mouse (female, 2 months old) was first immunized by an intra-peritoneal injection of an emulsion of 500 μg FITC-conjugated bovine serum albumin (FITC–BSA) with complete Freund's adjuvant. Three weeks after the first immunization, the mouse was immunized with the same dose of FITC–BSA in an emulsion with incomplete Freund's adjuvant as the second immunization. Four weeks later the mouse was immunized with the same dose of FITC–BSA in an emulsion with incomplete Freund's adjuvant as the third immunization. The antisera were prepared from 100 μl of whole blood by bleeding each week after the first immunization. The panels (a to f) show the patterns of anti-FITC antibodies separated by 2-D AEP with 4 μl of antisera at the days indicated below the panels using 10 μ*M* of FITC–DEX in the gels. The arrowheads indicate high affinity antibodies, and the closed triangles indicate low affinity antibodies. The asterisks indicate the antibodies induced by the second immunization. The antibodies were specifically stained by ELISA method as described in Fig. 8.

the course of immunization of the mice with FITC-conjugated bovine serum albumin (Fig. 12). Furthermore, we examined the effects of the carrier protein (Fig. 13) and the hapten array (Fig. 14) on the production of the antibodies [51]. The hapten array of FITC-conjugated dextran ($M_r = 50\,000$) generally suppressed the production of anti-FITC antibodies at secondary response. However, free hapten of fluorescein did not affect the production. Bovine serum albumin suppressed the production of antibodies with low affinity but not those with high affinity for the hapten. These results might indicate the epitopic suppression induced by immunization with carrier protein and the inactivation of hapten-specific B cells in vivo.

3.3. Microheterogeneity of monoclonal antibodies (mAb)

Myeloma proteins specific to dextran [39,52] and mAb derived from hybridoma cells specific to FITC and DNP [48,53] were separated by 2-D AEP into several IgG spots having an identical affinity to dextran and the haptens, respectively, but different p*I* values. Mimura and coworkers carried out detailed examinations on the mechanisms to generate the charge heterogeneity of mAb using two-dimensional gel electrophoresis of Fab and Fc fragments and in gel digestion of heavy and light chains of mAb spots by papain [53] as shown in Fig. 15 and by V8 protease [52]. The more acidic isoforms of the mAb

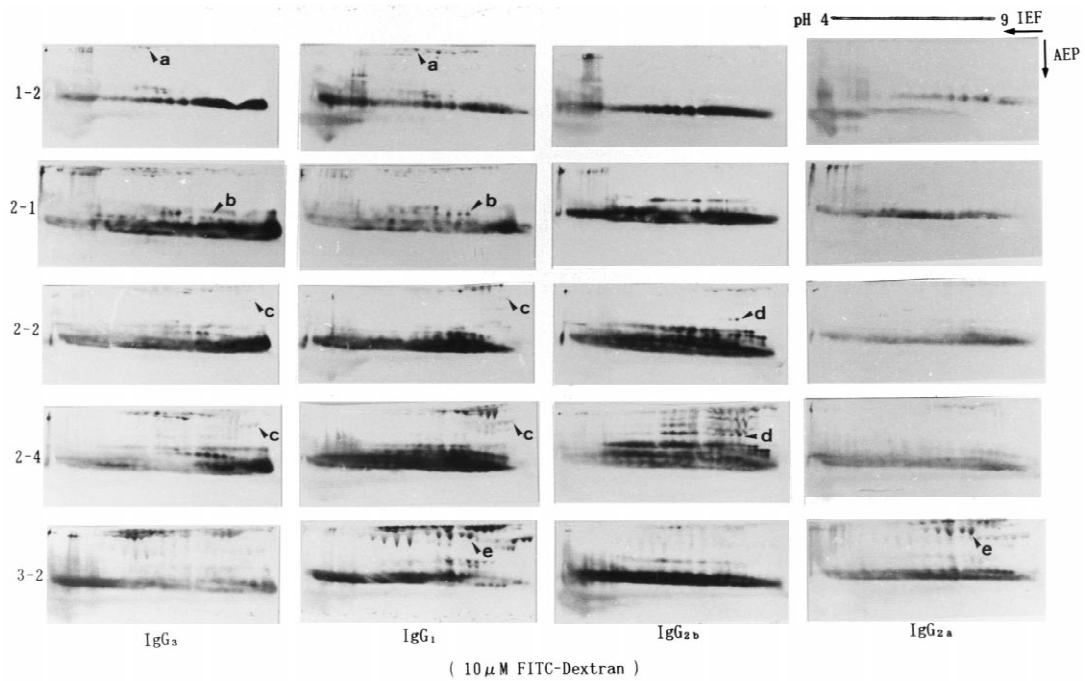

Fig. 12. IgG subclass switch during the immunization of a mouse [50]. The samples are 4 μl of the antisera used in Fig. 11. The antibodies separated by 2-D AEP were specifically stained by ELISA method as described in Fig. 9.

yielded more numbers of peptide fragments by V8 protease, and the incubation of the highly purified mAb at alkaline pH (pH 9) in sterilized buffer solutions increased the more acidic isoforms of mAb. Furthermore, they analyzed the charge heterogeneity of mAb derived from hybridoma cells, 1B7-11, which were treated with tunicamycin as a potential inhibitor of glycosylation of immunoglobulins [53]. The treatment with tunicamycin did not alter the charge heterogeneity. These results suggest that the charge heterogeneity of mAb is due to intra- and extra-cellular modifications of the polypeptide chains such as deamidation but not glycosylation. This may reflect molecular aging of antibodies.

4. Prospects on affinity electrophoresis

We describe here the principles and the applications of affinity electrophoresis to studies of immune response. In the early twentieth century, Tiselius applied a technique of electrophoresis for the separation of immunoglobulins (antibodies) in human serum. The technique triggered the development of electrophoretic techniques for separating biomolecules such as proteins, enzymes, and nucleic acids. Subsequently, the development of electrophoretic techniques for separating biomolecules via specific interactions with their affinity ligands, called ‘affinity electrophoresis’, enabled us to study biospecific interactions using small quantities of native samples. Furthermore, two-dimensional affinity electrophoresis made it possible to separate polyclonal antibodies into monoclonal antibodies and to study the diverisification and affinity maturation of antibodies specific to a hapten in a mouse during the immunization with the hapten-conjugated carrier protein.

Recently, a technique of capillary electrophoresis was developed for the separation of biomolecules in very small amounts of samples [54]. This technique has been used for analyses of dynamics of metabolism of pharmaceutical agents [55] and immunoreactions [56,57]. In the future, affinity techniques using high-performance capillary electrophoresis will

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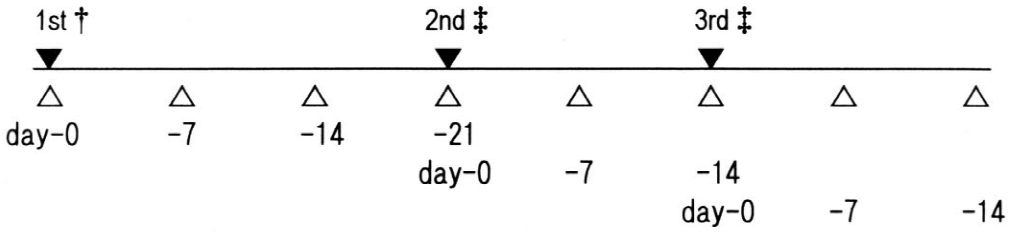

†: FITC-BSA (FITC:BSA=11:1)
‡: FITC-BSA or effectors(saline,BSA,HSA,FLU,FITC-DEX)
▼: 100μL of FITC-BSA or effectors in an emulsion with Complete or Incomplete Freund' Adjuvant was injected intraperitoneally.
△: 200μL of whole blood was taken by a puncture of the veniplex lining of an eye ball of a deeply anesthetized mouse.

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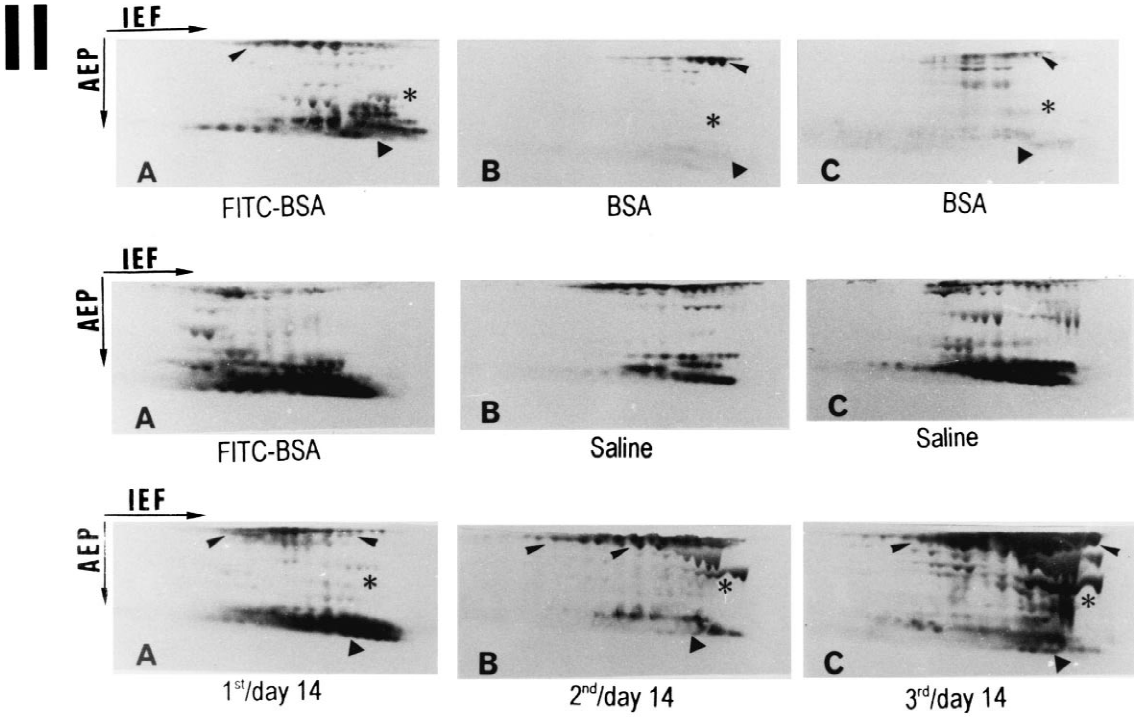

Fig. 13. Carrier induced epitopic suppression of anti-FITC antibodies analayzed by 2-D AEP [51]. The schedule of immunization of BALB/c mice with FITC–BSA followed by injections of the effectors of the carrier protein of BSA, FITC–BSA, free hapten of Fluorescein and the hapten array of FITC-conjugated dextran 50 (FITC–DEX) is shown in panel I. Panel II shows the alterations in 2-D AEP patterns of anti-FITC antibodies by the injections of BSA (upper panels), saline (middle panels) and FITC–BSA (lower panels). The panels A, B and C indicate 2-D AEP patterns of anti-FITC antibodies in antisera on day 14, 14 days after the first immunization with 500 μg of FITC–BSA (1st), on day 28, 14 days after the injections of the effectors (2nd) and on day 42, 14 days after the additional injections of the effectors (3rd). The arrowheads, closed triangles and asterisks indicate high, medium and low affinity antibodies, respectively.

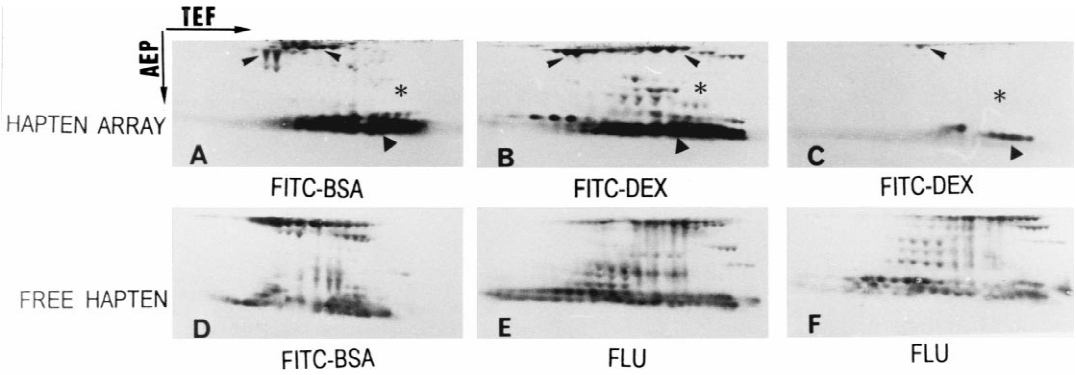

Fig. 14. Effects of a hapten array, FITC–DEX on the production of anti-FITC antibodies analyzed by 2-D AEP [51]. The schedule of immunization of the mice is the same as in Fig. 13. Panels A, B and C show the alterations in 2-D AEP patterns of anti-FITC antibodies in antisera by injections of FITC–DEX, and panels D, E and F show the alterations in 2-D AEP patterns by injections of a free hapten of fluorescein.

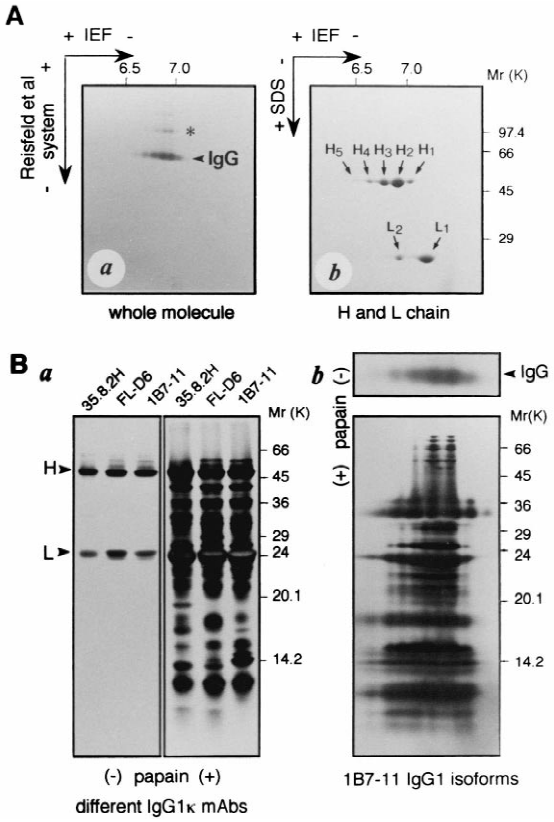

Fig. 15. Microheterogeneity of monoclonal antibodies analyzed by 2-D AEP and peptide mapping with papain [53]. Panel A shows the charge heterogeneity of monoclonal antibody, 1B7-11 (IgG1 κ) specific for DNP and of its heavy and light chains. Panel B (a) shows the peptide mapping of monoclonal antibodies (IgG κ) having different antigen specificity. Panel B (b) shows the peptide mapping of isoforms with different p*I* values.

be a useful tool for biochemical research and clinical diagnoses of metabolic diseases and critical care of patients.

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