Journal of Chromatography, 597 (1992) 345–350
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 950

Analysis of the interaction between an α(1→6)dextran-specific mouse hybridoma antibody and dextran B512 by affinity electrophoresis

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ABSTRACT

Carbohydrates are common environmental antigens. As dextran B512 is composed of a repeating structure of simple antigenic determinants, it is widely used to study the immunochemical properties of immunoglobulins. Two-dimensional affinity electrophoresis patterns of a mouse monoclonal antidextran antibody (35.8.2H; IgG_1, BALB/c) were produced to obtain insights into the microheterogeneity of the monoclonal antibody. The monoclonal antibody was separated into about six spots which had an identical affinity to dextran B512, but differed in their isoelectric points (p*I*). In addition, the pH dependence of the binding affinity of this antidextran to dextran B512 was examined. By comparing affinities obtained by affinity electrophoresis between weakly basic (pH 9.5) and weakly acidic (pH 3.8) discontinuous buffer systems, the latter showed an affinity about 500 times lower than the former. The change in the affinity was investigated with a continuous pH gradient by an affinity titration curve and was seen to change markedly at about pH 6. This suggests that the histidine at residue 34 in the light-chain CDR1 is largely responsible for the dextran binding.

INTRODUCTION

Dextran has been shown to be highly immunogenic in humans [1] and mice [2]. Dextran B512 is composed of 95% α(1→6)- and 5% α(1→3)-linked glucosyl chains [3–5], *i.e.* a repeating structure of simple antigenic determinants without a protein carrier portion. Therefore with the advent of hybridoma technology [6], monoclonal antidextran antibodies have been produced [7–11] to investigate the properties of antibody-combining sites specific for α(1→6)dextran, such as the sizes, shapes [12,13] and binding affinities [14]. Antidextran antibodies were shown to have two kinds of combining sites: one specific for the non-reducing ends of α(1→6) dextran chains, called a cavity-type site, and the other specific for internal linear portions, termed a groove-type site [12,15]. The V-region cDNA of the heavy and light chains of the antidextran antibodies were cloned and sequenced to correlate the immunochemical specificity and properties of these antibodies with their primary structures [11,16,17]. V-Region nucleotide sequences of these antidextrans have shown that antibodies with groove-type sites use V_κ genes belonging to the V_κ-OXL or the V_κ-ARS germline gene family and V_H genes belonging to the J558, the 36-60 or the J606 germline gene family [16,17], and that antibodies with cavity-type sites use the V_κ-W3129 genes and V_H genes belonging to the X24 or the Q52 germline gene family [11,18]. It has been shown that different V_κ and V_H germline genes can be used to produce similar combining sites [17].

In this study the amino acid sequences were related to the pH dependence of the binding affinities of the antidextrans, which were found to decrease markedly at low pH. This relationship was found using affinity electrophoresis (AEP) [19,20]. In this technique the binding affinity is quantified from a decrease in the migration of an antidextran by dextran B512 incorporated in the polyacrylamide gel. It was shown that the histidine at residue 34 in the

0021-9673/92/$05.00

light-chain CDR1 was responsible for the interaction between antidextran antibodies and dextran B512.

EXPERIMENTAL

Purification of mouse monoclonal antidextran antibodies

Mouse monoclonal antibodies specific for α(1→6)dextran in ascitic fluids were kindly provided by Dr. Elvin A. Kabat (Columbia University, USA). These antibodies were purified by affinity chromatography using a 1-ml Sephadex G50 column (15 mm × 6.5 mm I.D.), which was equilibrated with 0.02 *M* Tris-buffered saline (pH 7.5) (TBS). The ascitic fluid was applied to the column, which was then washed thoroughly with 200 ml of TBS overnight until no protein was detected with the spot test [21]. In this test 2 μl of eluate were spotted on a nitrocellulose membrane to stain with 0.08% Ponceau 3R in 7% acetic acid. Monoclonal antidextran antibodies were eluted from the column with 1% dextran 4 in TBS. About 100 μl of the eluate were collected in each tube and a total of about twenty tubes was needed. The protein content of 2 μl of the eluate was estimated with the spot test. Fractions containing protein were used for two-dimensional AEP without dialysis and, if not immediately required, were stored with 0.02% sodium azide at 4°C.

AEP and determination of apparent dissociation constants

AEP was performed in polyacrylamide disc gels in the same way described previously [19]: 5.1% T, 2.6% C [$T = (a + b) \times 100/m$ (%), $C = b100/(a + b)$ (%), a = acrylamide (g), b = N,N′-methylenebisacrylamide (g), m = volume of buffer] for the separating gel and 3.12% T, 20% C for the stacking gel with the discontinuous buffer system reported by Davis [22] and Ornstein [23] or Reisfeld *et al.* [24] to determine the apparent dissociation constants (K_d) at weakly basic pH or weakly acidic pH, respectively.

The separating gels contained a series of different concentrations of dextran B512 as a macromolecular affinity ligand for antidextran antibodies. The sample solution has the following composition: an ascitic fluid containing a monoclonal antidextran antibody, 0.0625 *M* Tris–HCl for the Davis [22] and Ornstein [23] system or 0.0625 *M* acetate–potassium for the Reisfeld *et al.* [24] system, 2% Sepaline, 5% sucrose and bromphenol blue [22,23] or methylene blue [24] as a tracking dye. Immunoglobulin (Ig) A and IgM were incubated with 30 m*M* mercaptoethanol for dissociation to their monomers for 1 h at room temperature. The AEP separation was run at 2 mA per tube until the tracking dye reached the bottom of the gel. After electrophoresis, the gel was removed from the glass tube and a fine wire was inserted at the position of the tracking dye band. The gels were stained overnight in 0.05% Coomassie brilliant blue R-250 in 7% acetic acid containing 50% methanol. The gels were electrophoretically destained in 7% acetic acid.

From the AEP patterns, K_d values were calculated according to the original affinity equation [20]:

$$1/r = 1/R_0 (1 + c/K_d)$$

where R_0 is the ratio of the migration distance of the band of the monoclonal antibody in the absence of dextran B512 in the control gel to that of the tracking dye front, and r is the ratio of the migration distance of the band of the monoclonal antibody in the presence of dextran B512 in the affinity gel at a concentration c to that of the tracking dye front.

Two-dimensional AEP of a monoclonal antidextran antibody (35.8.2H)

Two-dimensional AEP consists of capillary polyacrylamide gel isoelectric focusing (IEF) in the first dimension and polyacrylamide slab gel AEP in the second dimension [25]. The standard IEF gel had the following composition: 5% T, 3% C; Pharmalyte pH 4–6.5 (0.625%), pH 6.5–9 (0.625%) and pH 5–8 (1.25%); 10% glycerol; 1.5 m*M* lysine; 6 *M* urea; 0.077% N,N,N′,N′-tetramethylethylenediamine (TEMED) and 0.05% ammonium persulphate. The upper part of the capillaries were filled with an anodic electrolyte solution (0.04 *M* L-glutamic acid) and then 0.1–0.2 μg of the purified monoclonal antibody containing 30% sucrose was applied. For the cathodic electrolyte solution 1 *M* NaOH was used. IEF was run at 5°C at 250 V for 15 min, followed by 500 V for 15 min, 1000 V for 15 min and finally 2000 V for 2 h. The affinity gels were prepared in the same way as for AEP except that 77

× 84 × 1 mm slab gels were prepared instead of disc gels. The IEF gel was loaded on the top of the stacking gel where the stacking buffer was layered. The stacking buffer had the same composition as the buffer of the stacking gel with 5% sucrose and a tracking dye. Electrophoresis was run at 150 V until the tracking dye reached the bottom of the separating gel. After two-dimensional AEP the proteins were electroblotted onto a nitrocellulose membrane in the blotting buffer (50 m*M* glycine–HCl, pH 2.5) at 5 V for 2 h at 5°C. Goat anti-mouse IgG antiserum and rabbit anti-goat IgG peroxidase conjugated were used for the first and the second antibody, respectively, and the peroxidase substrate (a mixture of 60 mg of 4-chloro-1-naphthol in 20 ml of methanol, 60 μl of 30% hydrogen peroxide and 100 ml of TBS) was used to detect the monoclonal antibody. All chemicals used in electrophoretic separations were of analytical reagent grade purity or highest purity available and were obtained from different commercial sources.

Affinity titration curves

The affinity titration curve was determined in a modified version of the previously described method [26]. The IEF gel containing the affinity ligand was prepared with the following composition: 5% T, 3% C, 2.4% Sepaline (pH 3.5–10) 0.017% TEMED, 0.04% ammonium persulphate and the required concentration of dextran B512. After filling a gel mould (100 × 100 × 1 mm), the gel was polymerized in an incubator at 37°C for 1 h. The cathode and the anode electrolyte solutions were 1 *M* NaOH and 1 *M* H_3PO_4, respectively. The same electrolyte solutions were used for both the first and the second dimensions. The first dimension was run at 6.4 W for 55 min. After the electrode gel layers were cut away, the gel was turned through 90°, new electrode strips were overlayed on the margins of the gel, and the trench was filled with the sample (about 0.5 μg IgG in 20 μl TBS). Affinity electrophoresis was performed, perpendicular to the first dimension, at 600 V for exactly 20 min. Monoclonal IgG was detected with immunoblotting in the same way as in two-dimensional AEP.

RESULTS AND DISCUSSION

To investigate its microheterogeneity, a monoclonal antibody, 35.8.2H (IgG_1, BALB/c) was separat-

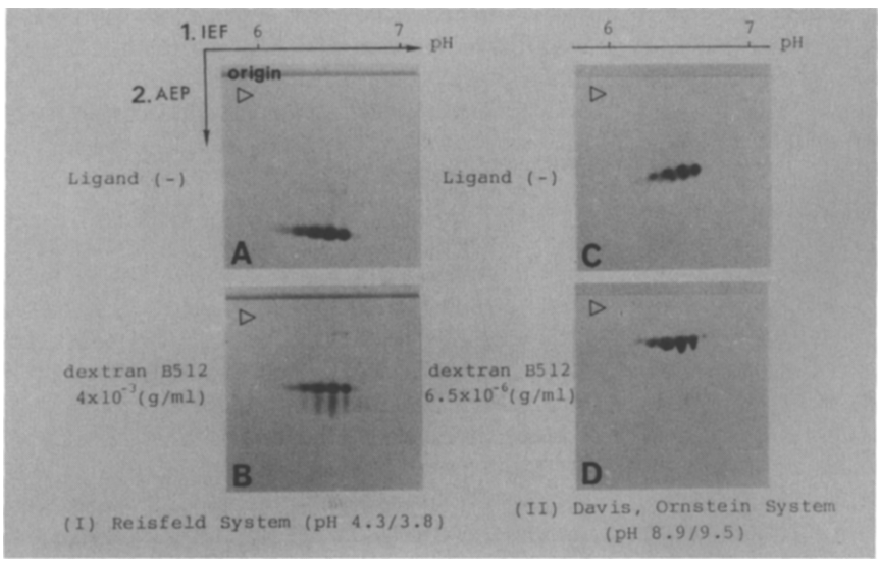

Fig. 1. Two-dimensional AEP patterns of monoclonal antibody 35.8.2H at two pH values. (A), (B) Two-dimensional AEP patterns of purified monoclonal antibody 35.8.2H (IgG_1, BALB/c) obtained by the Reisfeld [24] buffer system, pH 3.8, in the absence (A) and presence (B) of $4.0 \cdot 10^{-3}$ g/ml dextran B512. (C), (D) Two-dimensional AEP patterns of the same antibody obtained by the Davis [22] and Ornstein [23] buffer system, at pH 9.5, in the absence (C) and presence (D) of $6.5 \cdot 10^{-6}$ g/ml dextran B512.

ed by high resolution two-dimensional AEP. This antibody was separated into about six spots (Fig. 1) which had different p*I* values but showed an identical decrease in migration. This indicated that the spots had an identical affinity to dextran B512, which suggested that they had identical antibody combining sites. In our earlier study a group of IgG spots which had an identical affinity to a ligand but different p*I* values were suggested to be derived from a single antibody-producing cell line when a polyclonal anti-dinitrophenyl antibody was separated by two-dimensional AEP into several hundred homogeneous IgG spots [25]. The present result was consistent with our hypothesis. In other words, two-dimensional AEP makes it possible to separate a polyclonal antibody into monoclonal antibodies. Although this microheterogeneity of the monoclonal antibody may be due to post-translational modifications such as glycosylation and phosphorylation, our preliminary studies showed no effects of enzymatic treatments with endoglycosidase F and alkaline phosphatases.

There was another important difference between the patterns at the two pH values. With the system of Reisfeld *et al.* [24] a more than 100-fold greater concentration of dextran B512 was needed to detect retardation in the mobility of this antibody than was needed with the system of Davis [22] and Ornstein [23]. To compare K_d values at pH 9.5 and 3.8, affinity electrophoresis was performed (Fig. 2). As the concentration of dextran B512 increased, the band of the monoclonal antidextran antibody retarded. The K_d values of this antibody with dextran B512 at pH 9.5 and 3.8 were $8.0 \cdot 10^{-6}$ and $3.8 \cdot 10^{-3}$ g/ml, respectively. This indicated that the affinity of this antibody at pH 3.8 decreased to a 475th of that at pH 9.5.

To examine which amino acid residue was responsible for the pH dependence of the affinity, an affinity titration curve was obtained. This method made it possible to examine the pH at which the affinity changes by AEP with a continuous pH gradient in the range pH 3.5–10. In the control gel without dextran B512 (Fig. 3A), the affinity titra-

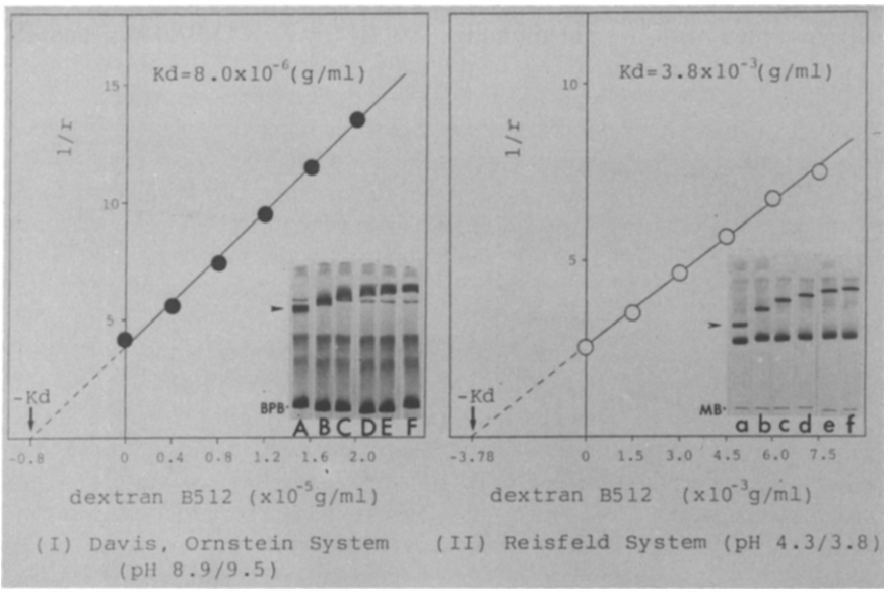

Fig. 2. AEP patterns and affinity plots of monoclonal antibody 35.8.2H at two pH values. Ascitic fluid containing monoclonal antibody 35.8.2H was electrophoresed in 5% polyacrylamide gels containing a series of concentrations of dextran B512 as a macromolecular affinity ligand by the Davis [22] and Ornstein [23] system, at pH 9.5 (I) and by the Reisfeld [24] system, at pH 3.8 (II). The monoclonal antibody band (arrowhead) was stained by Coomassie brilliant blue R-250. Concentration of dextran B512 (g/ml) I: (A) no ligand, (B) $0.4 \cdot 10^{-5}$, (C) $0.8 \cdot 10^{-5}$, (D) $1.2 \cdot 10^{-5}$, (E) $1.6 \cdot 10^{-5}$, (F) $2.0 \cdot 10^{-5}$; II: (a) no ligand, (b) $1.5 \cdot 10^{-3}$, (c) $3.0 \cdot 10^{-3}$, (d) $4.5 \cdot 10^{-3}$, (e) $6.0 \cdot 10^{-3}$, (f) $7.5 \cdot 10^{-3}$. Affinity plots were made according to the orginal affinity equation (see under Experimental). Abscissa indicates dextran B512 concentrations. Ordinate indicates reciprocal values of the relative migration distance ($1/r$). The intercept of the regression line on the abscissa gives a negative value of dissociation constant ($-K_d$).

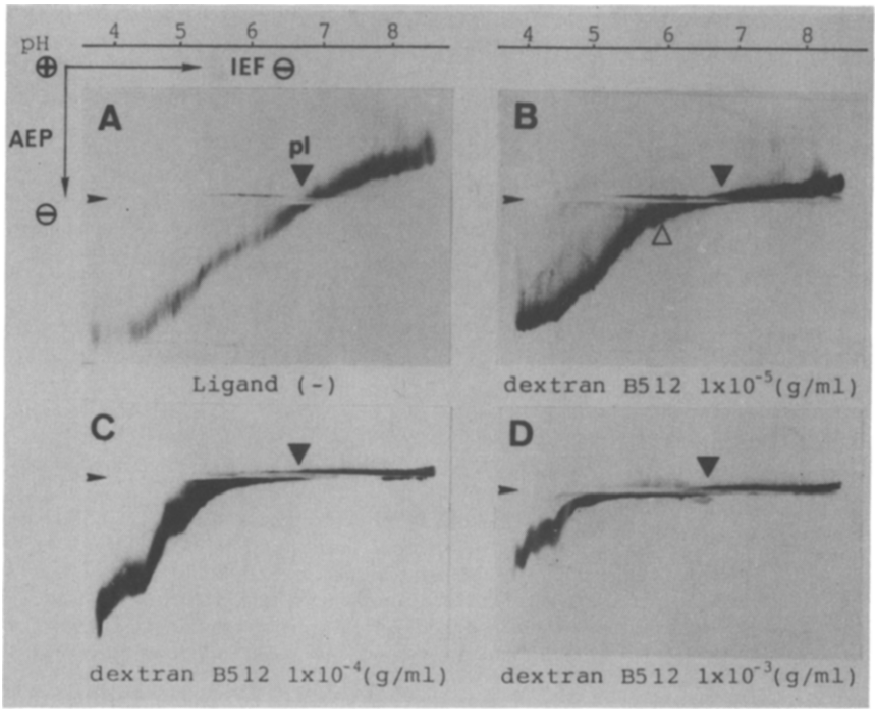

Fig. 3. Affinity titration curves of monoclonal antibody 35.8.2H. (A) No ligand, (B) $1.0 \cdot 10^{-5}$, (C) $1.0 \cdot 10^{-4}$ and (D) $1.0 \cdot 10^{-3}$ g/ml. Note that the position of the isoelectric point of this antibody (▼) remains the same. The change of affinity of the antibody is found at the position of the open triangle (△). The arrowheads show trenches for sample application.

tion curve was a sigmoid curve intercepting the sample application trench at pH 6.8 of this antibody's p*I* value. When the affinity gel contained $1.0 \cdot 10^{-5}$ g/ml dextran B512 (Fig. 3B), the curve showed a marked decrease in the migration distance of the antibody at high pH values (above 6) but no significant retardation at pH 4. This indicated that the affinity of this antibody to dextran B512 decreased at about pH 6. As the concentration of dextran B512 was increased, the mobility of this antibody at acidic pH values also gradually retarded. The antibody at pH 4 retarded in the presence of $1.0 \cdot 10^{-3}$ g/ml dextran B512 (Fig. 3D), which corresponded to the K_d value obtained with the system of Reisfeld *et al.* [24]. The plot of log K_d against pH (Fig. 4) showed that the affinity of this antibody changed markedly in the pH range 5.8–6.0. This strongly suggests that a certain histidine residue in the complementarity determining region (CDR) of this antibody was responsible for the pH dependence of the affinity.

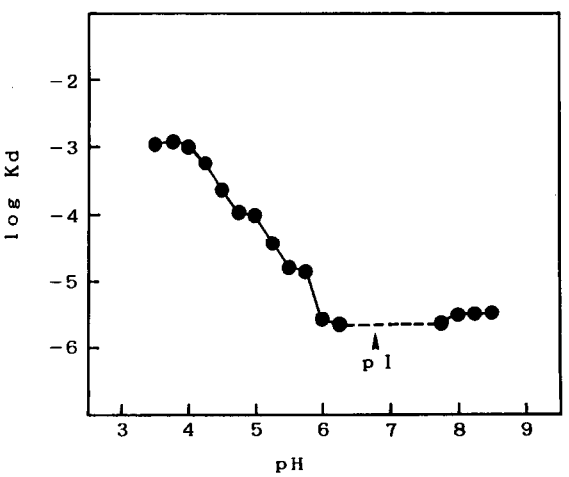

Fig. 4. Plot of log K_d against pH. The K_d values were obtained from affinity plots of relative migration distances of monoclonal antibody 35.8.2H at each pH. The relative migration distances were measured from affinity titration curve patterns of monoclonal antibody 35.8.2H at a series of concentrations of dextran B512.

The primary structure of the monoclonal antibody 35.8.2H has been deduced from the nucleotide sequence of its cDNA [11]. It shows that this antibody is encoded by V_κ-OXL and J558 for the light and heavy chain, respectively, and that it has only one histidine at residue 34 of the light-chain CDR1 in the amino acid sequence of the CDRs. According to other sequence data [16,17,27], this histidine is conserved in all anti-α(1→6)dextran antibodies encoded by V_κ-OXL. Although some anti-2-phenyloxazolone antibodies encoded by V_κ-OXL substitute another amino acid for this histidine and conserve the specificity, no antidextran antibodies are found to use such light chains. These facts support our hypothesis that this histidine residue plays an important role in the interaction between dextran B512 and antidextran antibodies with groove-type sites encoded by V_κ-OXL.

ACKNOWLEDGEMENT

We thank Dr. Elvin A. Kabat for kindly providing mouse monoclonal antidextran antibodies in ascitic fluids and crucial suggestions during this research.

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