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Conformation of Group "a" Epitope in Hepatitis B Surface Antigen

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Abstract □ To elucidate structure of group "a" epitope, mouse antibodies that express idiotype monoclonal antibody and anti-idiotype monoclonal antibody against the group-specific "a" determinant were purified by hydroxyapatite column. To obtain hepatitis B surface antigens (HBsAg), HBsAg positive blood was sequencially purified by ammonium sulfate precipitation, hydroxyapatite, sepharose 4B column chromatography and ultracentrifugation. The major protein (p25) and glycoprotein (gp30) of HBsAg were isolated by concanavalin-A sepharose 4B. The ability of p25-gp30 among the HBsAg to inhibit the idiotype-anti-idiotype reaction was dependent on conformation, since reduced and alkylated p25-gp30 virtually lost their inhibitory capacity when compared to native HBsAg. The data suggest that hepatitis B antigen is a conformational antigen critically dependent upon the disulfide bonds of p25-gp30.

Keywords □ idiotype antibody, anti-idiotype antibody, HBsAg, epitope, competitive ELISA inhibition.

Hepatitis is a public health problem of worldwide importance. Hepatitis B virus (HBV) infection is highly polymorphic, ranging from inapparent forms to acute hepatitis and severe chronic liver disease. Chronic liver disease can lead to cirrhosis, parenchymal liver failure and death. Thus acute and chronic hepatitis is major medical problems. In 1964, Blumberg *et al.* [1,2] identified hepatitis B virus. Australia antigen [Au (1)] was first detected in the serum of an Australian aborigine using as an antiserum a precipitating antibody which developed in the serum of a transfused patient. Au (1) had been identified as hepatitis B surface antigen (HBsAg). During HBV infection in humans, virus particles are present in very large quantities in the blood. In some forms of chronic infection, the serum contains only empty envelopes. Occasionally, it also contains complete virions, the empty envelopes always remaining in large excess. The presence of complete virions in the serum is indicative of an active viral multiplication in the liver. The empty envelopes consist of spherical or filamentous particles 22 nm in diameter. The virion of 42 nm diameter consists of an evelope and a nucleocapsid containing a circular DNA molecule, a DNA polymerase, a protein kinase activity and a DNA-linked protein. The envelope carries the hepatitis B surface antigen (HBsAg), whereas the capsid carries the hepatitis B core antigen (HBsAg). When the virions are present in the blood, an additional soluble antigen related to the capsid, the hepatitis B *e* antigen (HBeAg), is generally detected in the serum. The principal diagnostic marker of acute or chronic infection with HBV is the appearance in the serum of surface antigen (HBsAg), but in acute infection it does not remain in the serum during recovery. Soon after the discovery of the hepatitis B surface antigen and its association with viral hepatitis, HBsAg was shown to possess antigenically distinct subdeterminants. The first evidence concerning the he-

terogeneity of Au antigen was presented by Levene and Blumberg[3], who identified a common specificity "a" found in all Au^+ sera, and two other apparently alternative specificites. Multiple antigenic specificities of virus HB were later described by Le Bouvier[4] and several other groups of investigators[5-9]. Four antigenic specificities, besides the common antigenic determinant "a", are well documented. They are d and y of Le Bouvier[4] and w and r of Bancroft *et al*[7]. Thus four major subtypes, *i.e.*, adw, ayw, adr, and ayr, came into vogue. In 1973, Soulier and Courouce[8,10] defined the immunochemical heterogenicity of the "a" determinant into a1, a2, a3, and a4 with several intermediate specificities. Because the subtypes of "a" were not associated with r but only with w, the W.H.O. Committee on Viral Hepatitis recommended that the determinant w be designated w1, w2, w3, and w4 instead of the "a" determinant. With the recognition of subspecificities of the "a" determinant, at least 10 different subtypes of HBsAg were recognized, *i.e.*, ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adr, adyw, and adywr. Other subtypes x[4], t[11], q[9] and g, have been described, but these subtypes are less well defined as yet. As all HBsAg subtypes have group determinant "a", the "a" group antigenic determinant(s) is of particular medical interest because the anti-a antibodies induced by immunization with one HBsAg serotype confer protection against HBV infection with the other serotypes[13]. The possibility of utilizing a synthetic HBsAg peptide for the development of a HBV vaccine was raised in earlier studies[14,15]. Therefore, it is imperative that the epitopes associated with each peptide be defined in terms of the critical "a" antigenic determinant(s). To elucidate antigenic structure of group "a" determinant, an idiotype monoclonal antibody to group "a" determinant and an anti-idiotype monoclonal antibody to idiotype antibody variable region were isolated by hydroxyapatite column and group "a" determinant was isolated by Con-A sepharose column, followed by reduction and alkylation. Finally, idiotype-anti-idiotype competitive ELISA inhibition by group "a" determinant was carried out. This paper reports the results of mass production, purification, competitive ELISA and characterization of monoclonal antibodies and hepatitis antigens.

EXPERIMENTAL

Cells, reagents and instruments

6E and 4-8H hybridoma cells were kept in our laboratory. Cells were grown as suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco Grand Island, New York USA), 100 unit of penicillin/m*l*, and 100 μg of streptomycin/m*l* and 10 mM Hepes (Sigma, St. Louis, MO USA). HBsAg was purchased from Blood Center, Korea Red Cross. BSA, horseradish peroxidase, o-phenylene-diamine 2HCl, pristane, SDS, acrylamide, TEMED, Tris and molecular weight markers were purchased from Sigma (St. Louis, MO). Sepharose CL-4B, concanavalin A and hydroxyapatite were purchased from Pharmacia (Uppsala, Sweden). Serodia-HBs was purchased from Fujirebio (Osaka, Japan). Other chemicals and solvents used were of the first grade and were used without further purification. An immunoplate of 96 wells was purchased from Nunc (Roskide, Denmark). Cell culture petri dish was purchased from Falcon (Bellco Biotechnology Vineland, New Jergey, USA).

Ultrafiltration was carried out with membrane kit of Amicon (Danbers, USA). Optical density (OD) of ELISA was measured by automatic ELISA reader (Helsinki, Finland). Hybridoma cells were grown at 37℃, in a 5% CO_2 incubator (Vindon, U.K). Male Balb/c mice were provided by the Experimental Animal Farm of Seoul National University.

Cell culture

After the frozen vial was removed from the liquid nitrogen storage, the vial was thawed rapidly in 37℃ water bath, taken care to avoid contact with water on the seal area of the vial. The vial was then swabbed with 70% ethanol, dried and opened. After the cells were carefully removed from the cell suspension using a sterile Pasteur pipette, They were transferred to a centrifuge tube containing 10 m*l* of RPMI 1640 with 10% FBS at room temperature. The cells were immediately recovered by centrifugation at 1500 rpm for 5 min. After the cells were resuspended with a second 10 m*l* of RPMI 1640 with 10% FBS, They were transferred to a tissue culture dish and to a CO_2 incubator. The cells were subcultured for optimal growth.

Mass production of MAbs from mouse ascites

For ascites production, mouse was treated intraperitoneally with pristane for 3-4 weeks before use.

Hybridoma cells were twice washed with HBSS and resuspended to a final concentration of 2×10^7 per ml HBSS. This was injected at 0.5 ml per mouse (10^7 cells/mouse). When the tumor growth was maximal, mice were sacrificed by cervical dislocation and ascites was collected asceptically with a Pasteur pipette. The collected ascites was centrifugated at 1200 rpm for 10 min to remove cells.

Purification of MAbs

Hydroxyapatite chromatography: Each of 10 ml of ascitic fluid, 6E and 4-8H, was applied to a 1.8 cm×36 cm column of hydroxyapatite hydrated in column buffer A (0.01 M Na-phosphate, pH 6.8). The bound proteins were then eluted with a 300 ml of 0.01 M to 0.3 M linear phosphate gradient (pH 6.8) at room temperature. The flow rate was controlled with a peristaltic pump at 30 ml/h, and 5 ml fractions were collected. Columns were regenrated by washing with 3 bed vols. of 0.5 M phosphate buffer followed by 3 bed vols. of 1 N NaCl followed by 6 bed vols of column buffer A.

SDS-polyacrylamide gel electrophoresis: SDS-PAGE (vertical slab) was carried out on 3% stacking gel and 10% separating gel containing 0.1% SDS at room temperature as described by Laemmli[17]. The sample was mixed with 10 μl of sample buffer in a microcentrifuge tube heated for 2 min at 100℃ and then applied to the gel. After electrophoresis, the gel was stained in 0.25% coomassic brilliant blue dissolved in methanol, and destained in 25% methanol containing 7% acetic acid.

Conjugation of Horse Radish Peroxidase (HRPO) to immunoglubulin

Antibody-HRPO conjugate was prepared according to the method of Nakane[23]. Four mg of horse radish peroxidase was dissolved in 1 ml of distilled water and 200 μl of freshly prepared 0.1 M sodium periodate solution was added and stirred gently for 20 min at room temperature. After dialysis against 0.01 M sodium acetate (pH 4.4), 20 μl of 0.1 M sodium carbonate buffer (pH 9.5) was added to raise pH to approximately 9-9.5 and 1 ml of the IgG fraction was immediately added to be conjugated. After addition of 100 μl freshly prepared sodium borohydride solution (4 mg/ml in distilled water), the solution was left 4℃ for 2 hr. The solution was applied to a 33 cm×2 cm column of sephadex G-100 hydrated in PBS. The conjugate was then eluted with 100 ml PBS at 4℃. The flow rate was controlled with a peristaltic pump at 15 ml/hr and 2 ml fractions were collected.

Purification of HBsAg

HBsAg-positive blood was obtained from the Blood Center, and centrifugated at 3000 rpm in JA 10 rotor for 15 min at 4℃. The plasma was purified by modified Peterson's method.

Ammonium sulfate fractionation: To 500 μl of plasma, 88 μg of solid ammonium sulfate was added. The solution was stirred at room temperature for 30 min, then centrifugated for 30 min at 10,000×g. The supernatant was recovered and to each 100 μl, 12.7 μg of solid ammonium sulfate was added. The solution was stirred for 30 min at room temperature, then centrifugated at 10,000×g for 30 min. The precipitate was collected and dissolved in a minimum of water, and then dialyzed against 0.01 M potassium phosphate buffer, pH 6.8.

Hydroxyapatite chromatography: The dialyzed solution was applied to a hydroxyapatite column (4×20 cm). The column was equilibrated with 0.01 M potassium phosphate buffer pH 6.8 prior to use. The HBsAg was eluted with 0.1 M potassium phosphate buffer pH 6.8. The eluted solution was pooled and concentrated to 15 ml by using an Amicon ultrafiltration cell with a YM-100 membrane.

Sepharose 4B chromatography: The concentrated solution was applied to a sepharose-4B column (6×120 cm). The column was previously equilibrated with 0.1 M phosphate buffer, pH 6.8. Fractions of 8 ml were collected. HBsAg activity of each fraction was measured by RPHA method (Serodia-HBS, Fujirebio, Japan). Fractions of titer more than 200 were pooled. The pooled fractions were concentrated to 100 ml by pressure dialysis in an Amicon ultrafiltration cell with YM-100 membrane.

Ultracentrifugation: To concentrated solution 100 ml, 43.7 g of cesium chioride was added. The solution was made into homogeneous solution of cesium chloride, density 1.35 g/ml and centrifugated for 40 hrs at 60,000 rpm at 4℃. From each tube, 0.5 ml solution was fractionated and HBsAg activity was measured. Fractions of titer more than 1000 were pooled and then dialyzed against 0.01 M PBS at 4℃. The HBsAg activity was measured by RPHA method and the protein concentration was measu-

Purified HBsAg
↓
Distruption of HBsAg with triton X-100
↓
Fractionation of disrupted HBsAg on Con A-sepharose
↓
Elution of unbound polypeptides with washing buffer
↓
Elution of bound polypeptides with elution buffer
↓
Removal of trition X-100 Con A-sepharose fractions
↓
SDS-polyacrylamide gel electrophoresis

Scheme 1. Isolation of HBsAg polypeptides.

red by Lowry method.

Isolation of HBsAg polypeptides

Disruption of HBsAg with triton X-100: Purified HBsAg in 0.01 M tris-HCl pH 7.3, was disrupted by overnight incubation at 37℃ in the presence of triton X-100 and NaCl at final concentrations of 2% and 0.5 M, respectively.

Fraction of disrupted HBsAg on Con A-sepharose: A column 2×14 cm of Con A-sepharose 4B was equilibrated with 0.01 M tris-HCl pH 7.3 buffer containing 2% triton X-100, 0.5 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. Disrupted HBsAg was applied to the column which was then washed with the buffer to remove unbound material. The bound material was eluted with 0.01 M tris-HCl, pH 7.3, containing 2% triton X-100, 0.5 M NaCl and 5% α-methyl-D-mannoside (α-MM). The fractions (0.5 m*l*) were collected by monitoring the proteins by UV-monitor.

Removal of triton X-100 from Con A-sepharose fractions: The pooled fractions were first dialyzed extensively against 0.01 M tris-HCl, pH 7.3, at 4℃; remaining amount of triton X-100 were then removed by a modification of the method of Holloway[20]. Washed 'Biobeads' SM-2 (Bio-Rad Laboratories, Richmond, California) were pre-incubated for 3 hr at room temperature with 5% gelatin and washed in a sintered glass funnel. The beads were added to the triton X-100-containing solution at a concentration of 1 g/m*l* and the mixture incubated on a shaker for 45 min at room temperature. The solution was then separated from the beads. This procedure lowered the triton X-100 concentration to 0.02 %, without any significant binding of protein to the beads.

SDS-polyacrylamide gel electrophoresis: SDS-PAGE was carried out as stated above. This procedure was summarized in Scheme I.

Reduction and alkylation of purified p25-gp30 micells

Reduction and alkylation of p25-gp30 micells of HBsAg were carried out in phosphate buffered saline (PBS), pH 7.2, and in 4 M or 8 M urea, made up of 0.01 M tris (hydroxymethyl) aminomethane buffer, pH 8.2. The purified p25-gp30 micells were diluted to a protein concentration of 200 μg/m*l* as determined by the method of Bradford *et al.*(1976), dialyzed against either PBS, 4 M or 8 M urea, and reduced with 0.1 M-dithiothreitol (DTT) for 2 hr at 37℃. Both DTT reduced and unreduced control preparation were alkylated by the addition of dry iodoacetamide to a final concentration of 0.5 M. All samples were subsequently dialyzed against several changes of PBS and tested for antigenicity by competitive ELISA method.

Competitive ELISA

Reagents: (1) Glycine Buffered Saline (GBS, 0.1 M pH 8.31): Ten grams of NaCl, 7.3 g of glycine and 1 g of NaCl were dissolved in one liter of distilled water.

(2) Bicarbonate Buffer (BCB, 0.1 M pH 8.2-8.4): Eight point four gram of $NaHCO_3$ and 0.11 g of Na_2CO_3 were dissolved in one liter of distilled water.

(3) 2% Bovine Serum Albumin-Bicarbonate Buffer (BSA-BCB): After dissolution of 2 g of bovine serum albumin in 100 m*l* of BCB, this solution was filtered with millipore filter (0.22 μm).

(4) Enzyme Conjugate: Horseradish peroxidase was conjugated with anti-HBsAg MAb. The enzyme conjugated antibody was diluted to 1 : 800 in 0.1% BSA-PBS

(5) OPD Solution: OPD (o-phenylenediamine·2 HCl) tablet (OPD/tablet: 12.8 mg) was dissolved in 5 m*l* of diluent (0.02% H_2O_2 in 0.01 M phosphate citrate buffer, pH 5.0).

Procedure: A polystyrene microtiter plate was coated with anti-idiotype MAbs (10 μg/m*l*, 0.1 m*l*/well in 0.1 M GBS, pH 8.31) for 2 hr at 37℃ or overnight at 4℃. After washing three times with wash buffer (0.1 M PBS, pH 7.4), the plate was treated with 2% BSA-BCB solution for 2 hr at 37℃. After removal of blocking buffer, competitive inhibition was carried out by mixing 50 μ*l* of HRPO-MAbs with 50 μ*l*

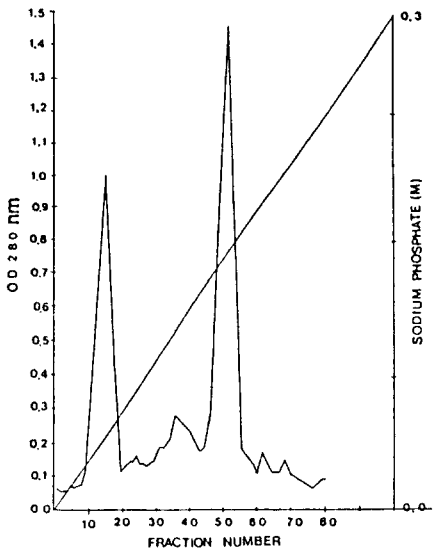

Fig. 1. Elution profile of mouse ascites fluid (6E) chromatographed on a hydroxyapatite column. Elution was carried out with linear gradient phosphate buffer between 0.01 M and 0.3 M. Each fraction was 4 ml.

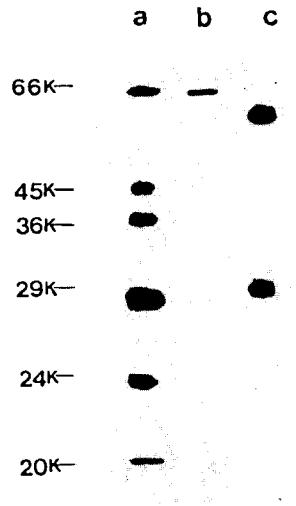

Fig. 2. SDS-PAGE analysis of column peaks detected by hydroxyapatite fractionation of 6E ascites fluid shown in Fig. 1. Lane a, molecular weight standards; Lane b, column peak 1; Lane c, column peak 2. Electrophoresis was carried out at 100 V for 1 hr and then 200 V for 2 hr.

of purified HBsAg which had been incubated for 30 min at 37℃. The concentration of HRPO-MAb was chosen to give a final OD of about 1.0 at 492 nm. One hundred μl of this mixture was added to the wells containing adsorbed anti-idiotype MAb and incubated for 2 hr at 37℃. After washing with the wash buffer, 100 μl freshly prepared substrate (OPD solution) was added into each well and incubated at room temperature at 30 min. The reaction was terminated by adding 100 μl of 1 N H_2SO_4. The color change of the substrate was determined by the automatic ELISA reader at 492 nm.

RESULTS

Purification of antibody

Purification of idiotype monoclonal antibody (6E): MAb 6E is IgG_1 antibody that recognized HBsAg group, "a" antigen. Ascites fluid containing MAb 6E was fractionated by hydroxyapatite chromatography on 1.8 cm×36 cm column and eluted with a 300 ml linear, 0.01 M-0.3 M phosphate gradient. The elution profile is shown in Fig. 1. Two major protein peaks were resolved. Samples from column peaks 1 and 2 were analyzed by SDS-PAGE. The gels were intentionally overloaded (30 μg/lane) to visualize any containing proteins. Column peak 1 contained albumin. In contrast column 2 contained immunoglobulin heavy and light chains (Fig. 2).

Purification of anti-idiotype monoclonal antibody (4-8H): MAb anti-idiotype antibody is IgG1 antibody that recognized hyper-variable region of idiotype monoclonal antibody. Ascites fluid containing MAb 4-8H was fractionated exactly as described above for MAb 6E and the profile was shown in Fig. 3. Two protein peaks were resolved. Samples from column peaks 1 and 2 were analyzed by SDS-PAGE. The gels were purposefully overloaded (30 μg/lane) to visualize any contaminating proteins. Column peak 1 contained albumin. In contrast column 2 contained immunoglobulin heavy and light chains (Fig. 4).

Purification of HBsAg

The yield of the purification of HBsAg was summarized in Table I. An overall yield of about 25% was obtained, based on reverse passive hemagglutination. The surface antigen displayed the typical UV absorption spectrum illustrated in Fig. 5. Maximal absorption was at 280 nm. The polypeptide

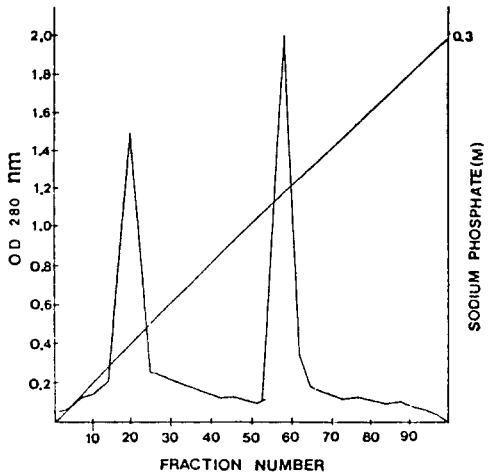

Fig. 3. Elution profile of mouse ascites fluid (4-8H) chromatographed on a hydroxyapatite column. Elution was carried out with linear gradient phosphate buffer between 0.01 M and 0.3 M. Each fraction was 4 ml.

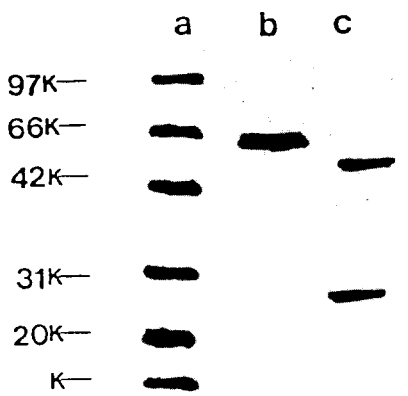

Fig. 4. SDS-PAGE analysis of column peaks detected by hydroxylapatite fractionation of 4-8H ascites fluid shown in Fig. 3. Lane a, molecular weight standards; Lane b, column peak 1; Lane c, column peak 2. Electrophoresis was carried out at 100 V for 1 hr and then 200 V for 2 hr.

composition of purified HBsAg was examined by SDS-PAGE (Fig. 7).

Isolation of p25 and gp30

HBsAg disrupted with 2% triton X-100 was fractionated on a column of Con A-sepharose in the presence of the same concentration of detergent. The unbound material was removed from the column by washing with the starting buffer. The arrow indicates the start of elution of the bound material with 0.01 M tris-HCl, pH 7.3, containing 2% triton-X-100, 0.5 M NaCl and 5% α-methyl-D-mannoside. The polypeptide composition of elution fraction by

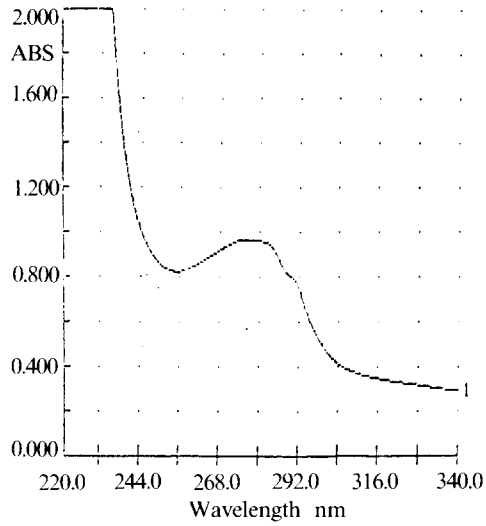

Fig. 5. UV absorption spectrum of purified B surface antigen.

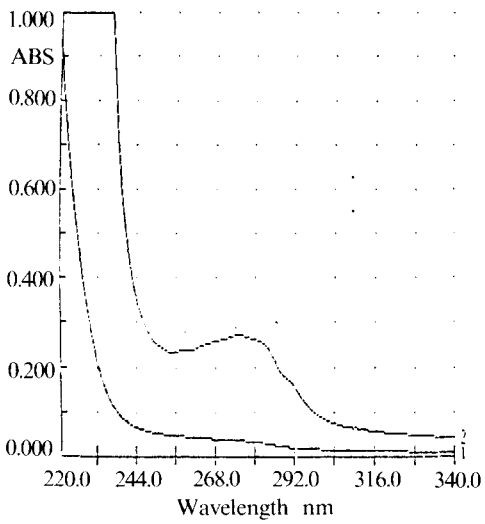

Fig. 6. UV absorption spectrum of the elution material after concentration.

Table I. Purification of HBsAg by the modified method of Peterson

Step	Volume (ml)	Titer[a]	Total activity (10^3)(mg/ml)	Protein[b]	Total protein (mg)	Yield (%)
1. Plasma	500	12,800	6,400	59.8	29,900	100
2. Ammonium-sulfate	220	25,600	5,632	56.0	12,320	88
3. Hydroxyapatite	750	6,400	4,800	9.15	6,863	75
4. Sepharose 4B	320	6,400	2,048	1.58	506	32
5. Ultracentrifugation	25	64,00	1,600	0.35	8.8	25

[a]Titer was defined as the reciprocal of the highest dilution (serial 2-fold dilution) capable of being detected by reverse passive hemagglutination (RPHA).
[b]Protein was determined by Lowry method.

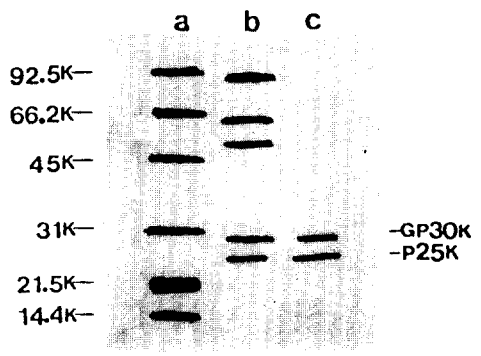

Fig. 7. SDS-PAGE analysis of the proteins comprising pooled fraction from Con A-Sepharose. Lane a, molecular weight standards; Lane b, purified HBsAg; Lane c, elution peak fraction of Con A-Sepharose. Electrophoresis was carried out at 15 mA constant current until the dye was eluted.

chromatography on Con A-Sepharose was examined by SDS-PAGE (Fig. 7). The α-MM eluate consisted of p25 and gp30 (Fig. 7).

Inhibition of the idiotype monoclonal antibody-anti-idiotype monoclonal antibody reaction by p25-gp30 micells

p25-gp30 micells were examined for the ability to inhibit the binding of the idiotype monoclonal antibody to its anti-idiotype monoclonal antibody. Reduction and alkylation of p25-gp30 micells destroyed antigenicity, either in the presence or absence of urea. In addition, treatment with iodoacetamide alone did not affect antigenic acitivity (Table II). These results suggest that conformation of antigenic determinant is critically dependent upon the disulfide bonds of protein moiety.

Table II. Inhibition of the reaction between the idiotype MAb and anti-idiotype MAb by p25-gp30 micells (OD 492 nm)

Treatment	Inhibition[a,b] (%)
Alkylation-PBS	100 (0.0)
Reduction and alkylation-PBS	1.7 (0.983)
Alkylation-4M-urea	99.1 (0.009)
Reduction and alkylation-4M-urea	1.0 (0.990)
Alkylation-8M-urea	99.6 (0.004)
Reduction and alkylation-8M-urea	(1.0)

[a]Percent inhibition $= 100 \times \left(1 - \frac{\text{OD bound with competing p25-gp30 micells}}{\text{OD bound without competing p25-gp30 micells}}\right)$
[b]Inhibition greater than 10% was considered significant

DISCUSSION

The development of synthetic peptides for possible utilization as animal virus vaccine has recently become an active area of investigation[25]. In addition to the work relating to HBV, several studies have been published on the properties of synthetic peptide analogue of influenza virus[26] and foot and mouth disease virus[27]. To develop an effective synthetic vaccine, it becomes apparent that basic knowledge relating to both the sequential and conformational properties of the critical antigenic determinants must be elucidated. To elucidate structure of group "a" epitope, we used inhibition of idiotype MAb-anti-idiotype MAb reaction instead of using the less specific techniques of immunodiffusion and counter electrophoresis. We reported that 6E idiotype MAb recognized group "a" epitope of

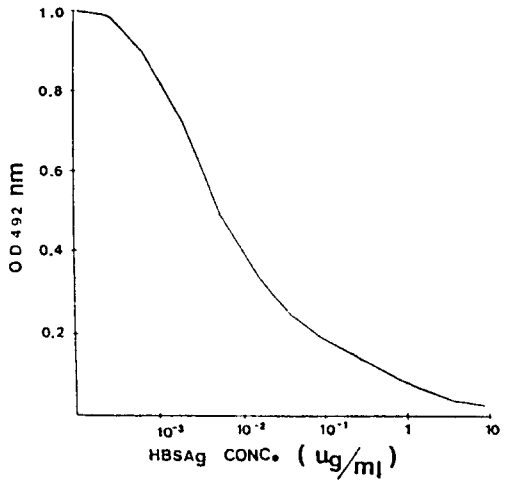

Fig. 8. Competitive binding assay of the idiotype-anti-idiotype by the purified HBsAg.

HBsAg[28]. Also we recently reported that 4-8H anti-idiotype MAb recognized hyper-variable region of 6E MAb[29]. After extensive purification, HBsAg derived from human plasma has been shown to contain two proteins, p25, major protein, and gp30, the glycosylated form of p25. After isolating intact p25-gp30 by concanavalin-A sepharose 4B, the reduction of p25-gp30 and alkylation of the free thiol groups abolished its reactivity that inhibited idiotype MAb-anti-idiotype MAb reaction, suggesting that group "a" epitope is conformation-dependent epitope. Cleavage of intramolecular disulfide bonds of a number of globular proteins resulted in a complete loss of reactivity with antibodies to native proteins. The role of disulfide bonds in stabilization of conformation-dependent epitopes has been reported for ribonuclease[30], lysozyme[31] and bovine serum albumin[32]. Similarities between our studies with p25-gp 30 that inhibited the idiotype MAb-anti-idiotype MAb reaction and those described with idiotype-anti-idiotype antiserum instead of monoclonal antibody[24] emphasize the role of comformation in maintaining the integrity of a given epitope.

CONCLUSION

Idiotype monoclonal antibody against the group "a" epitope and anti-idiotype monoclonal antibody which was mirror image of the group "a" epitope were purified by single step procedure using hydroxyapatite column chromatography. P25-gp30 micells isolated from HBsAg were reduced by DTT and alkylated by iodoacetamide in PBS, pH 7.2 and in 4 M or 8 M urea, made up of 0.01 M tris (hydroxyl methyl) aminomethane buffer, pH 8.2. P25-gp30 which was reduced and alkylated in PBS, 4 M and 8 M urea failed to inhibit idiotype MAb-anti-idiotype MAb reaction but p25-gp30 which alkylated in PBS, 4 M and 8 M urea inhibit idiotype MAb-idiotype MAb reaction. Consequently it has been shown that group "a" epitope is a conformational antigen critically dependent upon the disulfide bonds of the p25-gp30 polypeptide.

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