**DETECTION OF HBs ANTIGEN WITH LATEX PARTICLES COATED
WITH HUMAN ANTIBODY PREPARED BY AFFINITY
CHROMATOGRAPHY ON CONCANAVALIN A-SEPHAROSE***

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SUMMARY: Human HBs antibody was isolated by affinity chromatography on HBs antigen adsorbed to concanavalin A linked to Sepharose 4B. When a human anti-HBs immunoglobulin preparation obtained by Cohn's cold ethanol fractionation method was used as a starting material, the antibody was concentrated about 10 times in terms of the passive hemagglutination titer with a recovery rate higher than 50%.

Latex particles coated with human anti-HBs antibody thus prepared were proved to be useful in detecting HBs antigen in human blood samples. In its sensitivity and in rapidity of its performance, the antibody-coated latex agglutination test seems to be superior to conventional immunodiffusion techniques.

INTRODUCTION

Latex particles coated with antibodies of human and animal origin have widely been used for detecting or measuring corresponding antigens in test materials because of the rapidity and simplicity of its performance. Some workers tried to introduce this technique into the serological test for detecting HBs antigen in human blood (Leach and Ruck, 1971; Fritz and Rivers, 1972; Perkins et al., 1972) by using IgG of immunized animals as fractionated by physicochemical methods to coat latex particles.

Our first attempt at coating latex particles with human anti-HBs IgG prepared by Cohn's cold ethanol fractionation method was not successful, probably because of relatively low contents of the specific antibody in the IgG preparations (Miura et al., 1973). It was expected, therefore, that better results would be obtained if we further purify the anti-HBs antibody by such a specific immunological method as affinity chromatography and attach it to latex particles.

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The present report deals with the procedures we established for isolating HBs antibody and the demonstration of the usefulness of latex particles coated with the antibody.

MATERIALS AND METHODS

HBs antigen: HBs antigen was partially purified from human sera positive for HBs antigens by counter electrophoresis (CEP), pepsin hydrolysis, ultracentrifugation and gel filtration on Sepharose 4B (Pharmacia, Uppsala, Sweden) by a slight modification of the procedure described by Houwen *et al.* (1973).

Human anti-HBs antibody: The IgG fraction obtained by Cohn's cold ethanol fractionation method from donors' plasma positive for HBs antibody was used in most experiments; human serum containing HBs antibody was sometimes used without any fractionation.

Rabbit anti-concanavalin A serum: Antiserum to concanavalin A (Miles Laboratories, Kankakee, Ill.) was obtained by immunizing the rabbit three times each with 10 mg of concanavalin A mixed with Freund's complete adjuvant.

Serological tests: The passive hemagglutination test (PHA) for detecting anti-HBs antibody and the hemagglutination inhibition test (HI) for HBs antigen were performed as described by Vyas and Shulman (1970). Both single radial immunodiffusion (SRID) in Eizai plates purchased from Eizai Co., Tokyo, and radioimmunoassay (RIA, Aach, Grisham and Parker, 1971) with Ausria 125 kit (Dainabot, Tokyo) were carried out for detecting or measuring HBs antigen. Immunelectrophoresis and double immunodiffusion were performed in some experiments. Anticomplementary activity was measured by the test procedure given under the term 3.4.6 on Dried pepsin-treated human normal immunoglobulin of the Minimum Requirements for Biological Products (Ministry of Health and Welfare, 1973).

Purification of anti-HBs antibody: Concanavalin A-Sepharose (Con A-Sepharose, Pharmacia, Uppsala, Sweden) consists of Sepharose 4B activated by CNBr to which Con A was combined. One milliliter of the gel contains 8 mg of Con A. Hundred milligrams of partially purified HBs antigen was mixed with 10 ml of Con A-Sepharose and the mixture was continuously stirred overnight. Thereafter, unadsorbed components were removed by washing the gel with 0.01 M tris-HCl buffer containing 0.001 M CaCl_2 , 0.001 M MnCl_2 and 0.14 M NaCl (TCaMn). Then, 150 mg of anti-HBs IgG was added to the gel, and the mixture was gently stirred for 48 hr so that antibody could combine to the antigen adsorbed to Con A-Sepharose gel. This was then packed into a 1.0×15 -cm glass column and was washed with TCaMn. Continuous three washing cycles were necessary to remove noncovalently adsorbed protein and unstable Con A. Each cycle consisted of washing with 0.1 M glycine-HCl buffer (pH 4.5) and with 0.1 M tris-HCl buffer (pH 8.5).

Anti-HBs antibody was then eluted with 0.05 M glycine-HCl buffer containing 0.001 M CaCl_2 , 0.001 M MnCl_2 and 0.1 M NaCl at pH 3.5. After pH was adjusted to 7.2 with a concentrated glycine-NaOH solution, the eluate was dialyzed

against 0.14 M phosphate buffered saline (PBS, pH 7.2). Each fraction was tested by PHA for antibody activity; fractions positive for anti-HBs antibody activity were pooled and concentrated with Amicon Diaflo membrane XM 100 (Amicon Co., Lexington, Mass.).

Sensitization of latex particles: A stock suspension of latex particles (No. 15, 0.85μ , 4.1%) supplied by Takeda Chemical Industries Ltd. (Osaka, Japan) was diluted 1:10 in 0.2 M PBS (pH 7.2) and mixed with an equal volume of a purified antiHBs antibody solution. The mixture was incubated at 37 C for 100 min. Then, the coated latex particles were washed three times with PBS containing 0.02% polyvinylpyrrolidone (Daiichi Chem., Tokyo) and finally suspended in the buffer to about 1%.

Latex agglutination test: The test was carried out on a black plate. One drop of a test sample was mixed with one drop of the latex reagent (antibody-coated latex particles) on the plate by using a wooden stick. The plate was gently rocked for 2 min and examined macroscopically for the presence of visible agglutination of latex particles.

RESULTS

Purification of Anti-HBs Antibody

In our modification of the procedure of Houwen et al. (1973), the recovery rate of HBs antigen was about 10%. Therefore, about 700 ml of human serum positive for HBs antigen was used for obtaining HBs antigen to be adsorbed to 10 ml of Con A-Sepharose. Protein content of anti-HBs IgG used in this experiment was about 100 mg/ml and its PHA titer was 1:4096. When this anti-HBs IgG was used as starting material, antibody activity was detected from the 3rd to 44th fractions of 10 ml each, the highest PHA titer being 1:128 (Fig. 1). But the

Fig. 1. Titration of eluted anti-HBs IgG.

highest OD₂₈₀ value was only 0.07. On the other hand, when whole human serum positive for HBs antibody was applied to affinity chromatography without fractionation, the highest OD₂₈₀ value of the eluate found was as high as 1.2, but high antibody activity was not observed in any of the fractions.

Figure 2 shows the immunoelectrophoretic pattern of the purified anti-HBs antibody. As seen in (b), the antibody isolated directly from HBs antibody-positive serum formed several precipitation bands against anti-human whole serum antiserum, one of which could be identified as the one formed by the third com-

Fig. 2. Immunoelectrophoresis of eluted HBs antibodies.

- a) upper well: anti-HBs IgG
trough : rabbit anti-human whole serum
lower well: eluate from anti-HBs IgG
- b) upper well: eluate from HBs antibody-positive serum
trough : rabbit anti-human whole serum
lower well: HBs antibody-positive serum
- c) upper well: eluate from HBs antibody-positive serum
trough : rabbit anti-human IgG
lower well: eluate from anti-HBs IgG

ponent of complement (C3). Anti-HBs antibodies purified from anti-HBs IgG and serum were compared in (c) by immunoelectrophoresis against anti-human IgG serum, in which the latter formed a sharper arc of precipitation than did the former indicating that the latter had been less denatured.

The concentration of the antibody purified by affinity chromatography and contamination of the eluate with HBs antigen are shown in Table I. From the anti-HBs IgG preparation, antibody was further concentrated about eight times

TABLE I

Recovery of HBs antibody

Test materials	Protein contents	Volume	PHA*titer	HBs antigen		
				SRID*	HI*	RIA*
Anti-HBs IgG	100 mg/ml	1.5 ml	4096	—	—	—
Diluted (20×)	4.7		256			
Eluate	4.2	1.7	2048	—	—	—
HBs antibody-positive serum	65	50	64	—	—	n.d.*
Eluate	7.5	3.5	128	—	—	+

* PHA : Passive hemagglutination

SRID : Single radial immunodiffusion

HI : Hemagglutination inhibition

n.d. : Not done

in terms of the PHA titer and its recovery rate was over 50%. Furthermore, no HBs antigen was detected by SRID, nor by HI, nor by RIA. On the other hand, in the case of HBs antibody-containing serum, the concentration of antibody was only twice and its recovery rate was about 10%. Moreover, HBs antigen was detected in it by RIA.

No agglutination of human erythrocytes that could be attributable to the presence of Con A was observed in any of the eluates; but a marked precipitation band was formed in double immunodiffusion against rabbit anti-concanavalin A serum by the eluate when serum was used as starting material. As it is said that human erythrocytes are agglutinated by Con A at a concentration not lower than 3 μ g/ml, Con A below this level must have been present in the eluate in this case. Thus, HBs antigen might be present in the eluate not as free antigen but in the form of Con A-HBs antigen-antibody complexes.

Anticomplementary activity of the antibody obtained from the whole serum by affinity chromatography was roughly equivalent to that of the IgG fraction prepared by Cohn's fractionation method alone. However, when the concentrations of both the materials were so adjusted to give approximately the equal PHA titers the former was never found more anticomplementary than the latter.

Latex Agglutination Test (LAT)

The results of the latex agglutination test using latex particles coated with

various concentrations of purified HBs antibody are shown in Table II. Fairly good results were obtained within the range of antibody concentration from 0.1 to 0.25 mg/ml. This corresponds to the ratio of one volume of purified antibody solution (1 mg/ml) to 16 to 40 volumes of the latex suspension. When rabbit anti-human serum (Ortho, Raritan, N. J.) was used instead of a test sample, a positive result was also obtained. But with latex particles coated with 0.1 mg/ml of anti-HBs antibody, agglutination by an HBs antigen-positive sample was stronger than the reaction with anti-human serum, as shown in Fig. 3.

Comparative titration by LAT, SRID and HI on a panel of four plasmas positive for HBs antigen by CEP was undertaken. From Table III one can see that LAT is four to 10 times more sensitive than SRID and roughly equivalent to HI.

TABLE II

Latex agglutination test with latex particles coated with various concentrations of HBs antibody

Samples	Purified HBs antibody (mg/ml)							
	1.0	0.5	0.25	0.1	0.05	0.025	0.01	0.005
HBs antigen-positive plasma	+	+	++	++	—	—	—	—
HBs antigen-negative plasma	—	—	—	—	—	—	—	—
Rabbit anti-human serum	++	++	++	+	+	+	+	+

Fig. 3. Demonstration of latex agglutination test.

TABLE III

Comparative sensitivities on HBs antigen-positive plasmas

Plasma	Reciprocal titer		
	SRID*	HI*	LAT*
15C-2860	16	256	256
16A-1944	2	16	8
16A-7731	16	256	128
18A-1075	64	512	256

* SRID : Single radial immunodiffusion

HI : Hemagglutination inhibition

LAT : Latex agglutination test

DISCUSSION

As herein described, it was possible to purify the HBs antibody by affinity chromatography beyond the level that could be attained by a physicochemical fractionation process alone.

Affinity chromatography first described by Cuatrecasas, Wilchek and Anfinsen (1968) was applied to isolation of HBs antigen by Tripatzis and Horst (1971). Since the discovery of subtypes of HBs antigen (Le Bouvier, 1971; Bancroft, Mundon and Russel, 1972), this technique has also been used for isolating subtype-specific antibody by absorbing antiserum with the antigen of a particular subtype and eluting the antibody therefrom (Hollinger et al., 1973; Miyakawa, Imai and Mayumi, 1975). We utilized affinity of HBs antigen to aerosil for removing the antigen from human sera, but soon found that aerosil was not a suitable agent to be used for purification of HBs antigen (Ishiyama, Yasuda and Ito, 1974), although it was used for the purpose of purifying HBs antibody by Traavik (1975).

Cawley (1972) noticed that HBs antigen forms precipitate with concanavalin A, a protein obtained from jack bean. This substance was then used by Neurath, Prince and Lippin (1973) in affinity chromatography for isolation and purification of HBs antigen. Our procedure described in this report is its further application, in that specific HBs antibody was separated from the antigen which had been adsorbed to concanavalin A by their method.

In our procedure, in which Con A was used to bridge between the HBs antigen and Sepharose 4B, the fixation of HBs antigen was much firmer than in the method of attaching HBs antigen directly to Sepharose 4B, and the purity of the antigen in the applied material seemed less critical in the former than in the latter method. On the other hand, difficulties were always encountered with the step of eluting the antibody combined to the antigen. Molecules of Con A were very susceptible to low pH and a high salt concentration of the eluting buffer (Olson and Liener, 1967; Agrawal and Goldstein, 1967). When the pH was below 3.0, Con A tended to disintegrate into monomers which would appear in the eluate. From our repeated trials under various conditions, the best results were obtained with the eluting buffer of 0.1 M, pH 3.5 so far as Con A-Sepharose was used. Even in this case, repeated washing of the column with buffers of pH 4.5 and 8.5 prior to the elution seemed to be desirable to obtain consistent results.

Anti-HBs antibody purified from the IgG fraction in the present work seems unique in its recovery rate and in absence of contamination with HBs antigen. The present results with anti-HBs IgG as starting material seem fairly satisfactory in respect to the recovery of antibody and to the exclusion of contamination with HBs antigen. However, immunoelectrophoretic analysis revealed slight denaturation of IgG molecules, which must be inherent to the fractionation process. Nevertheless, the most antigen-binding activity was found to have been retained when adsorbed onto the surface of latex particles. It should be more desirable to use an IgG fraction obtained by such a procedure that

would cause as little denaturation as possible to be applied to affinity chromatography.

Another advantage of this method was the use of latex particles, which were found very stable, as was stated by Matsuzawa *et al.* (1973).

Although we can not state definitely that our antibody-coated latex reagent can be routinely used in screening blood donors, this technique, so often abandoned in the past because of instability of the reagents used, should be reevaluated for its usefulness.

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