Modified Counterelectrophoresis Method for Subtyping Hepatitis B Antigen

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A modified counterelectrophoresis (CEP) method was developed for determining the *d* and *y* subtypes of hepatitis B antigen (HB_sAg). In this method, HB_sAg of known subtype was diffused into the agarose gel before electrophoresis from the wells which were subsequently to receive the subtyping antiserum. This served to absorb the common anti-*a* antibody from the subtyping antiserum, which was heterologous with respect to the *d* or *y* component, during electrophoresis. The remaining *d* or *y* antibody then reacted type specifically with the antigen to be subtyped. The modified CEP method was much more sensitive than the immunodiffusion (ID) method for subtyping HB_sAg. Sixty-two sera which could not be subtyped by ID were successfully subtyped by the CEP method. The geometric mean HB_sAg complement fixation titer determined on 48 of these sera was significantly lower ($P = 0.016$) than that of a group of sera which could be subtyped by ID. Sixteen other sera could not be subtyped by either the CEP or the ID procedure (geometric mean titer = 5.2). Therefore, more sensitive means of subtyping must be used for some HB_sAg-positive sera.

Hepatitis B antigen (HB_sAg) is usually subtyped by immunodiffusion (ID) (5). However, experience in our laboratory as well as elsewhere (1, 7) has shown that this method is not sensitive enough for subtyping many HB_sAg-positive sera. Holland et al. described a counterelectrophoresis (CEP) technique for subtyping in which monovalent antisera prepared by appropriate cross-absorption procedures were used (4). They reported that some sera with HB_sAg undetectable by ID could be subtyped by CEP. A modified CEP technique for subtyping the *d* and *y* components of HB_sAg is described here which involves inhibition of precipitation due to the common *a* antibody component of subtyping antisera by prediffusing antigens of heterologous subtype into the gel. In effect, the *a* antibody is absorbed during electrophoresis by the antigens of heterologous subtype which were prediffused into the gel. The *d* or *y* antibodies of the subtyping antisera are left to react type specifically with the antigens being tested. Subtyping results obtained by this modified CEP method are compared with those obtained by ID.

MATERIALS AND METHODS

CEP. CEP was carried out on glass slides (8.2 by 10.1 cm) covered with 10 ml of 1% agarose in veronal buffer (1.84 g of diethyl barbituric acid, 10.30 g of sodium diethyl barbiturate made to 1 liter with distilled water; pH 8.5). One batch of agarose (Sea

Kem, Marine Colloids, Inc.) with a relative electrophoretic mobility value of -0.16, determined essentially as described by Wieme (9), was used throughout the experiments. Slides were prepared the day before an experiment was to be done and were stored overnight at 4 C. On the day of test, two rows of 10 holes, 5 mm in diameter and 6 mm apart (edge to edge), were cut in the center of the agarose layer on each slide. Slides were used in pairs for subtyping the sera positive for HB_sAg. For one slide of each pair, the wells on the anodic side were filled with a known *ay* antigen (complement fixation [CF] titer of 256). For the second slide of each pair, the wells on the anodic side were filled with a known *ad* antigen (CF titer of 512). The known *ay* and *ad* antigens were purchased from the American National Red Cross Blood Research Laboratory. The subtype of these antigens was confirmed here with reagents obtained from other investigators. Slides were incubated for 18 to 24 h at 35 C in petri dishes with moistened filter paper in the bottom to allow the known antigens to diffuse out of the wells. When these antigens had completely diffused out of the wells, sera to be subtyped were placed in the wells on the cathodic side of both slides of the pair (each serum was put in a well occupying the same position on the two slides). Hepatitis B antibody against the *ad* subtype was then used to fill the anodic wells on slide 1 of each pair, and hepatitis B antibody against the *ay* subtype was placed in the anodic wells of slide 2. Each antibody was used at a dilution determined by preliminary experiments as described in the results section. The antibodies used were (i) NIH interim reference reagent guinea pig hepatitis B antibody no. V801-501-058 prepared against purified *ad*

antigen and (ii) guinea pig hepatitis B antibody prepared against purified *ay* antigen (provided by W. Adrian Chappell, Viral and Rickettsial Products Branch, Center for Disease Control). Electrophoresis was carried out at room temperature with a Gelman power supply and chamber. Terry cloth wicks and the veronal buffer referred to earlier were used. A constant current of 25 mA was passed across the long dimension of the slides for 1.5 h. Slides were read with oblique lighting against a black background. Known *ad* and *ay* antigens were included on one pair of slides in each day's tests to serve as controls.

ID. Sera tested by the CEP subtyping technique were also tested by an ID subtyping method. Slides (7.5 by 5.0 cm) were covered with 6 ml of 0.5% agarose in tris(hydroxymethyl)aminomethane-buffered saline, pH 8.0 (5). The batch of agarose was the same as that used for the CEP tests. Slides were prepared the day before a test was to be performed and were stored overnight at 4°C. Holes were cut in the agarose layer on the day of test. A standard seven-well pattern (six peripheral wells and one central well) was used. Wells were 3 mm in diameter and 2 mm apart (edge to edge). The unknown serum to be subtyped was placed in alternate peripheral wells in this pattern with antigens of known subtype (both *ad* and *ay*) in the remaining peripheral wells. Antibody prepared against an antigen of known subtype was used in the central well. The same antisera were used as in the CEP method. Dilutions were usually 1:16 for the *ad* antiserum and 1:2 for the *ay* antiserum. Slides were incubated overnight at room temperature. The subtype of the unknown antigen could be determined by the pattern of reactions of identity or partial identity which developed with the known antigens, if the unknown antigen gave sufficiently strong ID reactions.

CF. CF tests were carried out by a micro procedure (3). Hemolysin was titrated by the plateau method with each batch of sheep cells. Complement was titrated each day that a test was carried out. Four 50% hemolytic units of complement were used in the test. Reaction mixtures were incubated in the micro plates at 4 to 6°C for 15 to 18 h. Approximately 8 U of NIH interim reference reagent guinea pig antibody (no. V801-501-058) prepared against purified *ad* antigen was used for determining the HB_sAg CF titers of the sera.

RESULTS

Determination of antibody dilutions. Preliminary experiments were carried out with the modified CEP technique to determine an appropriate dilution of each subtyping antiserum for use in the test. Several antigens of known subtype and varying CF titers were tested against dilutions of the two guinea pig subtyping antisera after prediffusion of the *ad* and *ay* antigens as indicated previously. For each antiserum, a dilution was selected which gave good lines of precipitate between the wells with all the antigens of homologous subtype but which gave

only minimal precipitates at the edge of the anodic wells with the antigens of the other subtype. On the basis of these experiments, a dilution of 1:20 was chosen for the *ad* antiserum and a dilution of 1:2 for the *ay* antiserum. Dilutions of antiserum were made in normal guinea pig serum to avoid the occurrence of nonspecific precipitates sometimes seen when buffered diluents are used in CEP (8).

Comparison of CEP to ID for subtyping. The types of reactions observed with the modified CEP subtyping method are illustrated in Fig. 1. Single lines of precipitate were seen between the reactant wells. For a given serum, such a line was seen on only one of the two slides of each pair. Results obtained with antigens of known subtype showed that this precipitate was due to the reaction of the *d* or *y* components of the antigens being tested with the *d* or *y* antibodies in the subtyping antisera. Accordingly, the subtype of the unknown antigens was indicated by the antiserum which gave this reaction. Small amounts of precipitate (not shown in Fig. 1) were sometimes seen around the edge of the anodic (antibody) wells along the arc facing the cathodic (antigen) wells. Presumably this was due to the reaction of the *a* component of the antigen of heterologous subtype placed in the wells the day before with the

FIG. 1. Results obtained with modified CEP method for subtyping HB_sAg. (a) Slide 1: reaction of unknown sera with *ad* antibody after prediffusion of *ay* HB_sAg. Lines between pairs of wells at positions 1, 2, 3, 4, 6, and 7 indicate these unknown antigens to be subtype *ad*. (b) Slide 2: reactions of unknown sera with *ay* antibody after prediffusion of *ad* HB_sAg. Lines between pairs of wells at positions 5, 8, 9, and 10 indicate these unknown antigens to be subtype *ay*. On both slides, the anodic (antibody) wells are to the right and the cathodic (antigen) wells to the left.

anti- α antibody in the antisera used in the test.

A total of 79 HB_sAg-positive sera were subtyped by the CEP method (Table 1). Fifty-six were subtype *ad* and 23 were subtype *ay*. With the ID procedure, 11 of the 79 antigens were definitely subtyped and six gave doubtful results. The remaining 62 specimens gave very weak precipitation reactions and could not be subtyped by ID. In all cases where the antigens could be subtyped by both methods or where ID gave doubtful results, the two procedures agreed on the subtype involved. Sixteen additional sera that were found positive for HB_sAg by the CF test could not be subtyped by either CEP or ID.

CF titers. HB_sAg CF titers were determined for 76 of the 95 sera on which subtyping was attempted. Table 2 shows the geometric mean titers of these sera arranged in groups according to the subtyping results obtained with CEP and ID. The CEP method allowed subtyping of antigens with CF titers which were significantly lower (*t* test, $P \approx 0.016$) than those of the antigens that could be definitely subtyped by ID (group C compared with group A, Table 2). Those sera which could not be subtyped by either CEP or ID had the lowest geometric mean titer of any group. This titer was significantly lower ($P = 0.001$) than that of sera which could be subtyped by CEP but not by ID (group D compared with group C, Table 2).

DISCUSSION

Bjorklund (2) described ID studies in which specific inhibition of precipitation was accomplished by prefilling wells in the reaction pattern with appropriate reagents. In his experiments, either antigen or antibody was placed in certain wells of the ID pattern for 3 consecutive days and allowed to diffuse into the gel. Inhibition of precipitation was observed when reactants homologous to the prediffused reagent were subsequently added to appropriate wells. Actually, precipitation was considered to have occurred at the edge of the wells as the reactant added last diffused out of the wells and met the reagent which had been prediffused into the gel. Data were presented to show that inhibition of precipitation by the prediffusion technique did not interfere with the formation of typical lines of precipitate by antigen and antibody heterologous to the prediffused reagent.

In the present experiments, a similar technique was used to inhibit precipitation of the common α component of the test HB_sAg by the anti- α antibody in the subtyping sera. The anti- α antibody reacted during electrophoresis with the α component of the antigens of heterologous subtype which had been prediffused into the

TABLE 1. Results of subtyping HB_sAg in 79 sera by CEP and by ID

Group	Subtyped by CEP		
	Subtype <i>ad</i>	Subtype <i>ay</i>	Total
Definite subtyping by ID	6	5	11
Doubtful subtyping by ID	1	5	6
Not subtyped by ID	49	13	62
Totals	56	23	79

TABLE 2. Geometric mean HB_sAg CF titers of sera tested for subtype by CEP and by ID

Group	No. of sera	Geometric mean CF titer
A. Definite subtyping by CEP and ID	6	45.3
B. Definite subtyping by CEP, doubtful by ID	6	25.4
C. Definite subtyping by CEP, not subtyped by ID	48	14.1
D. Not subtyped by CEP or ID	16	5.2

agarose layer from the anodic wells. The anti- α antibody migrating out of the anodic well met the antigen of heterologous subtype migrating back toward the anode and reacted with it at the edge of the anodic well. The *d* or *y* antibodies that remained then migrated further from the anodic well and formed a line of precipitate with the *d* or *y* components of the test antigens moving from the cathodic wells. This interpretation of the results is supported by the fact that only a single line of precipitate was observed between the reactant wells, that these lines appeared on only one of the slides of each pair, and that the subtypes established by the CEP method were the same as those determined by the ID procedure for all cases where the antigen was strong enough to be subtyped by ID. In effect, as pointed out by Bjorklund (2) for his ID experiments, reaction of the anti- α antibody with the α component of the antigen of heterologous subtype during electrophoresis is the equivalent of absorption of the HB_sAg typing antiserum with HB_sAg of heterologous subtype. Inhibition of precipitation by prefilling the anodic wells with antigen of heterologous subtype as described here would be expected to be as effective as it is in ID, if not more so, because the absorbing antigen would move back toward the anodic well during electropho-

resis and would be concentrated there.

The results presented in Table 1 clearly show that the modified CEP method is more sensitive for subtyping HB_sAg-positive sera than the ID procedure. Sixty-two antigens which could not be subtyped by ID were subtyped by CEP. Holland et al. (4) also indicated that their CEP procedure made possible the subtyping of sera with HB_sAg not detectable by ID. Comparison of the CF titers of the sera studied here showed that the modified CEP technique allowed subtyping of antigens with titers which were significantly lower than those of the antigens that could be subtyped by ID (group C compared to group A, Table 2). However, other sera with even lower CF titers could not be subtyped by either CEP or ID (group D, Table 2). Therefore, more sensitive means of subtyping must be used for some HB_sAg-positive sera.

In comparison to the CEP subtyping procedure described by Holland et al. (4), the modified method presented here is somewhat simpler. The necessity for preparing monovalent antisera against the *d* and *y* antigens by separate absorption procedures and for checking the absorbed antisera to be sure of the presence of excess HB_sAg is eliminated. The technique of diffusing appropriate antigens into the gel before electrophoresis in a manner similar to that described here may have application to the study of other antigen-antibody systems by the CEP method.

The *w* and *r* antigenic determinants characterized by Bancroft et al. (1) were not dealt with in this study. However, the guinea pig antisera used were determined in retrospect to have been prepared with *adw* and *ayw* antigens. Despite this fact, no reactions were observed in the subtyping experiments which could be attributed to *w* antibodies. Apparently the *w* antibodies, if present in these antisera, were absorbed out by the antigens of heterologous subtype during electrophoresis (i.e., the absorbing antigens were fortuitously *adw* and *ayw*). Because HB_sAg found in the United States usu-

ally contains the *w* determinant (1), the chance for such absorption to have occurred would have been very good. In their radioimmunoassay subtyping experiments, Ling et al. (6) similarly did not encounter any reactions which they related to the *w* determinant, although their guinea pig antisera were found to have been prepared with *adw* and *ayw* antigens. Likewise, Holland et al. (4) in their CEP subtyping experiments did not observe any reactions other than those attributable to the *a*, *d*, or *y* determinants. Thus, *w* antibodies, if present in the antisera used by these investigators, were apparently removed by their absorption procedures.

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