A Counterimmunoelectrophoresis Inhibition Test for the Detection of Hepatitis-Associated Antigen (36156)

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The use of counterimmunoelectrophoresis (CIEP) as a diagnostic and research technique for the rapid detection of viral (1–3) bacterial (4), and cancer related antigens (5) has become well established. CIEP has the inherent disadvantage of prozone phenomena due to antigen–antibody imbalance, and it is less sensitive than complement fixation (6). For the above reasons CIEP could produce erroneous results when used as a diagnostic screening procedure.

We describe below a counterimmunoelectrophoresis inhibition test (CIEPI), which is not susceptible to the prozone phenomena, and which is more sensitive than CIEP for the detection of hepatitis-associated antigen (HAA).

Materials and Methods. Test antigens and antiserum. Rabbits were immunized with purified HAA (Electronucleonics Laboratories, Inc., Bethesda, MD). The immune serum was absorbed with a pool of normal human plasma obtained from 6 donors (7). The absorbed antiserum did not produce a precipitin line on CIEP or gel diffusion with normal human plasma or serum. A single precipitin line was observed on CIEP or gel diffusion when HAA-containing plasma or serum was used as the antigen. When the Division of Biological Standards (National Institutes of Health, Bethesda, MD) first Reference Hepatitis-Associated Antigen (Australia Antigen) Panel of 3 HAA positive and 3 HAA negative samples of human serum was studied, the antiserum detected HAA in all 3 HAA-positive samples on CIEP, and did not produce a precipitin line with any of the 3 HAA-negative samples.

Samples of human plasma and serum were obtained from Dr. B.S. Blumberg (The Institute for Cancer Research, Fox Chase, Philadelphia, PA), and Princeton Hospital, Princeton, NJ.

CIEP Detection of HAA. Counterimmunoelectrophoresis was done on 4 × 4 in. glass plates covered with 13 ml of 0.9% agarose in 0.1 *M* barbital buffer, pH 8.2. Parallel columns of 4 mm diameter wells were cut 4 mm apart (edge to edge) in the agarose. The wells in the column near the anode (antibody wells) were filled with 0.017 ml of rabbit anti-HAA serum. Patient's serum or plasma (0.017 ml) was placed in the wells near the cathode (antigen wells). After electrophoresis (35 mA for 1.5 hr), the agarose covered glass plate was rinsed with water and examined for precipitin lines between the antigen and antibody wells. The appearance of a precipitin line indicates the presence of HAA in the patient's serum.

CIEPI Standardization. Rabbit anti-HAA serum (0.01 ml) was diluted in varied volumes (0.2 to 0.6 ml) of normal human plasma and incubated at 37° for 30 min. A box titration of the dilutions of rabbit antiserum and saline dilutions of an HAA-positive human plasma was done by CIEP (Table I). One unit of antiserum was defined as the greatest dilution of the rabbit antiserum that produced a precipitin line. The least amount of the HAA-positive plasma that produced a precipitin line with 1 unit of antiserum was defined as one unit of HAA; 1.5 units of HAA were used in all of the CIEPI tests.

CIEPI Detection of HAA. Rabbit anti-HAA serum (0.01 ml) was premixed with patient's serum or plasma and incubated at 37° for 30 min. The antibody wells were then

TABLE I. CIEP Box Titration of HAA and Anti-HAA.[a]

Dilution of HAA positive human plasma	Vol (ml) of normal human plasma mixed with 0.01 ml of rabbit anti-HAA				
	0.2	0.3	0.35	0.4	0.45
1:8	—	—	—	—	—
1:16	+	—	—	—	—
1:32	+	+	+	+	—
1:48	+	+	+	+	—
1:64	+	+	+	+	—
1:128	+	+	—	—	—
1:256	—	—	—	—	—

[a] + = precipitin line; — = no precipitin line.

filled with 0.017 ml of antiserum–patient serum mixture. The antigen wells were filled with 0.017 ml of the standardized HAA (1.5 units of HAA). After electrophoresis (35 mA for 1.5 hr) the agarose-covered glass plate was rinsed with water and examined for the presence or absence of precipitin lines between the antigen and antibody wells. If HAA is in the patient's serum, it combines with the rabbit antibody and inhibits the formation of a precipitin line with the standardized HAA preparation. The presence of a precipitin line indicates HAA is not in the patient's serum.

A control was used without rabbit anti-HAA to detect human samples that contained antibody to HAA; none were found in this study.

Results and Discussion. A panel of human sera and plasma samples (14 were positive for HAA by complement fixation, and 107 were negative) was examined by CIEP and CIEPI (1.5 units of antiserum). All 14 positive samples were detected by CIEPI; 12 of the 14 known positive samples were detected by CIEP. There were no false-positive reactions with either test.

The sensitivity of CIEPI using 4, 2, and 1.5 units of antiserum was compared with that of CIEP by the duplicate titration of an HAA-positive plasma. CIEPI was twice as sensitive (1.5 units of antiserum), as sensitive (2 units of antiserum), and ½ as sensitive (4 units of antiserum) as CIEP for the detection of HAA (Table II). Similar results were obtained when 5 HAA-positive samples of varied CIEP titers were tested by CIEPI

TABLE II. HAA Titer of 01003 with CIEP and CIEP Inhibition.

Method	Amount of rabbit antiserum (units)	HAA titer
CIEP	Box titration	100
CIEP inhibition	1.5	200
	2.0	100
	4.0	20

TABLE III. Comparison of the Detection of Known Complement Fixation HAA-Positive and HAA-Negative Samples of Human Plasma with CIEP and CIEPI.[a]

	+ or — by CIEP box titration	CIEP reciprocal titer	+ or — with counterimmunoelectrophoresis inhibition test, using antiserum (units):	
			2	1.5
HAA positive				
21142	—	—	—	+
12908	+	32	+	+
02979	+	128	+	+
12802	+	2	+	+
77650	—	—	—	+
HAA negative				
02353	—	—	—	—
0344	—	—	—	—

[a] + = HAA detected; — = no HAA detected.

using 1.5 and 2 units of antiserum (Table III). The sensitivity of CIEPI is inversely related to the concentration of the antiserum.

Schmidt and Lennette (8), using gel diffusion and complement fixation for the detection of HAA, stressed the importance of doing box titrations of anti-HAA serum and serum containing HAA to eliminate false-negative reactions due to either antigen or antibody excess. A similar procedure should be used to avoid false-negative reactions when CIEP is used, since CIEP is susceptible to prozones (6). In contrast, due to the nature of inhibition tests, CIEPI is not susceptible to false-negative reactions caused by antigen excess. In addition, since CIEPI can detect lower concentrations of HAA than CIEP (Table II), CIEPI provides the researcher with a simple method of detecting antigen over a broader range of concentrations than CIEP.

When CIEPI is used, controls should be used to detect antibody in the patient's serum, which may react with the standardized HAA. If a precipitin line is produced in this control, the antibody should be characterized to determine if it is anti-HAA. This added procedure is important since the presence of human anti-HAA may indicate HAA is present in the serum in an undetectable complexed form (9), and thus the serum may be capable of transferring the hepatitis agent.

Agar gel precipitin inhibition techniques (10) have been described that are more sensitive than agar gel diffusion and avoid the prozone and anticomplementary problems of complement fixation. The present study indicates that an inhibition test modification of CIEP can similarly be utilized.

Summary. A new technique, CIEPI, is described for the rapid detection of HAA. CIEPI is not susceptible to prozones due to antigen excess, and it is more sensitive than CIEP for the detection of HAA.

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