ELECTROOSMOPHORETIC RADIOIMMUNOASSAY: APPLICATION TO HEPATITIS-ASSOCIATED ANTIGEN

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Blumberg's discovery of the Australia antigen or hepatitis-associated antigen (HAA) and the subsequent demonstration of its relationship to serum hepatitis was a major contribution in the search for a method to eliminate carriers of serum hepatitis as potential blood donors (*1,2*). In a recent study of patients who had received blood transfusions that were serologically positive for HAA, three-fourths developed serum hepatitis, serum HAA, or an antibody response. Of those patients who had received blood that was serologically negative for HAA, 6% developed hepatitis (*3*). Possible explanations are that the techniques used for detecting HAA lacked either sensitivity or specificity for variants of the hepatitis etiological agent. It has been estimated that current, practical methodologies employed for the detection of HAA identify less than 50% of the carriers (*4*).

Immunoelectroosmophoresis (IEOP) has achieved the most widespread use for screening prospective blood donors for HAA (*5*). Although IEOP lacks sensitivity for the detection of all sera containing HAA, it does lend itself to mass screening because of its speed (1 hr or less) and economy. Other investigators have applied radioimmunoassay (RIA) techniques to the detection of HAA and anti-HAA (*6–9*). Their methods are very sensitive, approximately 2–4 orders of magnitude greater than that of the IEOP procedure. The major disadvantages of RIA techniques for mass screening have been time of analysis (1–6 days) and the cost of the required technological expertise. We have developed two rapid, sensitive radionuclide procedures for the detection of hepatitis associated antigen and antibody that combine the speed and simplicity of IEOP with the sensitivity of RIA.

MATERIALS

The following materials were used:

1. ^{125}I-labeled HAA: HAA* was labeled with ^{125}I (carrier-free*) by the method of Hunter et al (*10*) and purified according to the method of Walsh et al (*6*).
2. Antibody solution: Serum was obtained from patients with antibody to HAA, and diluted with sodium barbital buffer (0.05 *M*, pH 8.6) to a titer sufficient to bind 33% of the ^{125}I-HAA (*11*).
3. Control sera: Known serum specimens (by IEOP) contained either HAA, anti-HAA, or were negative for both.
4. Agar gel plates: Projector slide cover glasses (3¼ × 4 in.)† are coated with 10 ml of 0.8% solution of agarose‡ (*12*). Seventy-two wells can be cut in each plate, eight wells across and nine down. The wells measure 4 mm in diameter and are placed on 10-mm centers.
5. Electrophoretic equipment: Gilman electrophoretic chamber§ and an S-S voltage regulated power supply|| are used.
6. Ponceau red: Used as a visual monitor of electrophoretic migration, i.e., adequate time and milliamperage (coulombs).

METHOD

Single well procedure. In the test for HAA, procedure was as follows:

1. Five microliters of ^{125}I-antigen are added to the well.
2. Five microliters of unknown serum are added to the same well.
3. Five microliters of antibody solution are added to the same well.

* Obtained from Electro-Nucleonics, Bethesda, Md.

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* Cambridge Nuclear Billerica, Mass. 01821.

† B-324, Eastman Kodak Co., Rochester, N.Y.

‡ Bausch and Lomb, Sea-Kem.

§ Model 51170-1, Gilman Co., Ann Arbor, Michigan.

|| Model EA-2, Carl Schlucher and Schuell Co., Keene, New Hampshire.

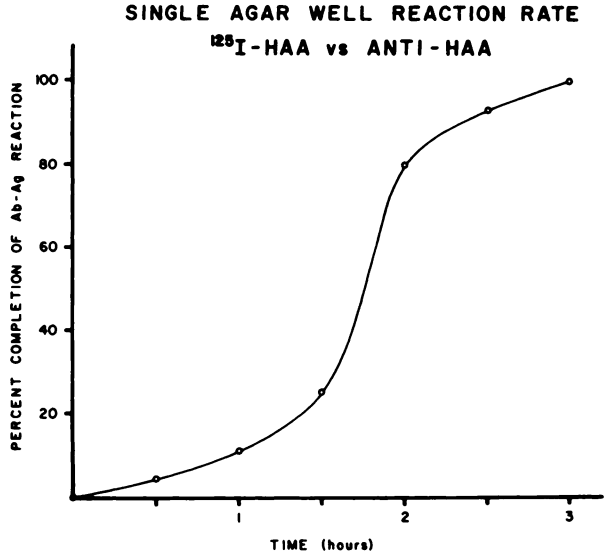

FIG. 1. Five microliters of labeled antigen (HAA) and 5 μl of antibody positive serum were added to individual wells at 30-min intervals. Incubation time for each is shown in hours. After incubation electrophoresis was applied for 1 hr. Bound and unbound labeled antigen were measured for each well.

4. Allow at least 3 hr for complete reaction at 25°C (see Fig. 1).
5. Fill all wells with sodium barbital buffer.
6. Electrophoresis is achieved in 1 hr with a constant current of 20 mA. Ponceau red migrates 2.5 in. from points of application along cathode edge of the plate.
7. The distribution of ^{125}I-HAA is determined by scintillation well counting of agarose sections.* The "bound" ^{125}I-antigen is separated from the "free" by dividing the agar on a line 1 mm on the anode side of the well. These agar blocks are counted separately in a scintillation well counter. For detecting HA antigen the percentage bound varies inversely as the serum antigen concentration (again in the presence of antigen excess).

In the test for hepatitis-associated antibody, the procedure was as follows:

1. Five microliters of ^{125}I-antigen are added to the well.
2. Five microliters of unknown serum are added to the same well.
3. Allow 3 hr for complete reaction at 25°C.
4. Follow Steps 5–7 of the single well method for HAA. For detecting HA antibody, the percentage bound varies directly with antibody concentration (in the presence of antigen excess).

For the simultaneous determination of HAA and anti-HAA, the following applies:

1. The procedure is identical to that for HAA.
2. Calculate percentage bound:
   a. If binding of ^{125}I-HAA exceeds the normal serum control, anti-HAA is present in the unknown serum. This represents noncompetitive protein binding.
   b. If binding of ^{125}I-HAA is less than the control, HAA is present in the unknown serum. This represents competitive protein binding.

Double well procedure. In the test for HAA, the double well procedure was as follows:

1. Five microliters of ^{125}I-antigen (10,000 cpm) are added to the cathode well.
2. Five microliters of unknown serum are added to the same cathode well.
3. Five microliters of antibody solution are added to the anode well.
4. Allow 10 min for reagents (i.e., steps 1–3) to diffuse into the agar.
5. Follow Steps 5–7 of the single well method for HAA. The antigen and antibody migrate toward each other, react, and form a stationary complex in the reaction zone. The block of agar between the two wells is removed and the activity is measured in a scintillation well counter.

In the test for hepatitis-associated antibody, the procedure was as follows:

1. Five microliters of ^{125}I-antigen are added to the cathode well.
2. Five microliters of unknown serum are added to the anode well.
3. After reagents diffuse into agar, follow Steps 5–7 described for the test for HAA, single well procedure.

In order to demonstrate the distribution of the ^{125}I-antigen after electrophoresis, the agar plates were exposed to 70-mm radiographic film.* Autoradiography was used to map the distribution of the labeled antigen as well as for graphic displays and to clarify the procedure. Autoradiography is not used for quantitative procedures. Quantitation is accomplished either on a deep well gamma counter or with an instrument we have designed for this purpose. With such special equipment, counting is accomplished without transfer of agar sections.

RESULTS

After electrophoresis for 1 hr the results obtained with the double well method for both antibody posi-

* Packard Auto-Gamma Spectrometer, Model 3001.

* DuPont, Cronex, SF2, x-ray, Spot film 70 mm, unperf., Wratten No. 1 or No. 2 safelight.

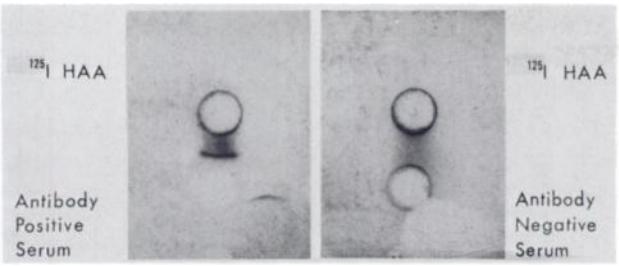

FIG. 2. Double well procedure. Results with positive and negative sera for HA antibody demonstrated by autoradiography.

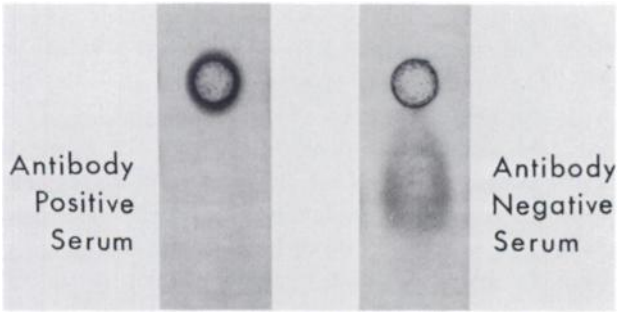

FIG. 3. Single well procedure. Results with positive and negative sera for HA antibody and ^{125}I-HAA antigen demonstrated by autoradiography.

tive and negative sera are illustrated by means of autoradiography (Fig. 2). With antibody positive serum (high titer) a well-defined precipitation line of radioactivity is found between the two wells. No ^{125}I-HAA is seen between the line of precipitation and the antibody well which is barely visible in this photograph. With antibody negative serum there is no line of precipitation. The ^{125}I-HAA migrates toward the antibody well and accumulates around the margin. The concentration of radioactivity in the precipitin zone varies directly as the level of antibody titer and the level of radioactivity within the zone varies inversely as the antigen titer in the presence of a constant antibody titer.

Figure 3 illustrates results obtained with the single well method. With antibody positive serum (high titer) the radioactivity is confined to the well after incubation and electrophoresis. With antibody negative serum the ^{125}I-HAA migrates toward the anode and appears as a broad trail of activity.

The quantitative relationship of the antigen-antibody reaction by the single well method was determined by serial dilutions of the antibody. After electrophoresis the bound ^{125}I-HAA within the well and the free ^{125}I-HAA in the agar were separated by dividing the agar on a line 1 mm from the edge of the well on the anode side. The radioactivity in the two parts of the agar was measured in a scintillation well counter and the amount of activity within the agar well expressed as the percent of the total. With antibody negative serum, 10% of the ^{125}I-HAA remained within the well and was considered as the residual background level. The relationship between serial dilutions of the antibody and the bound ^{125}I-HAA remaining in the well is shown in Fig. 4. One hundred percent competitive inhibition for HAA positive control serum has been demonstrated by means of the single well RIA technique.

The most significant factors influencing electroosmophoretic separation of the "bound and free" is that it was necessary to use the agar specified in this protocol. An additional carefully controlled reagent was the anti-HAA solution used for testing HAA. The concentration of the serum and its immunological properties largely control the sensitivity of the assay.

DISCUSSION

Since the infective agent(s) for serum hepatitis has not been grown in culture, current methodologies are all based on immunological reactions. Immunodiffusion (ID) was the first method to gain widespread use (*2*). Increased sensitivity and reduced time for detection of HAA were the result of the development of IEOP (*5*).

A further increase in sensitivity was offered by complement fixation techniques, but this procedure also increases the complexity of the procedures (*13*). Initially, hemagglutination (HA) and hemagglutination inhibition (HAI) methods for detecting HAA were suitable only for research applications (*14*); however, a recent report has described substantial improvement in stability of this technique (*7*). The reported sensitivity of HA is comparable to radioimmuno methods and the time required for analysis

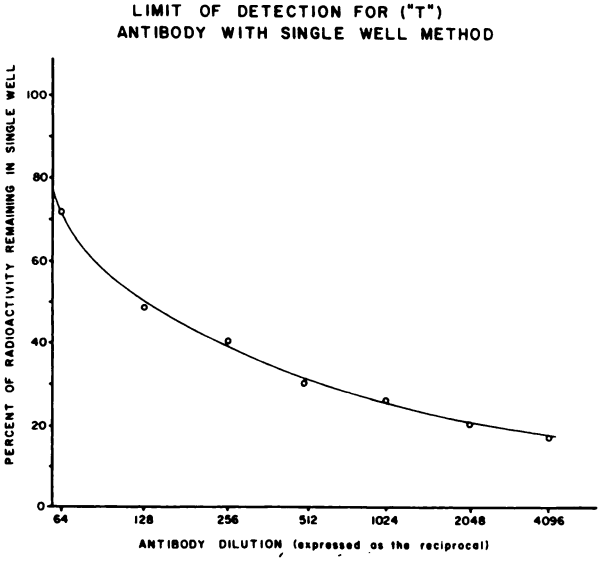

FIG. 4. Limit of detection for ("T") antibody with single well method.

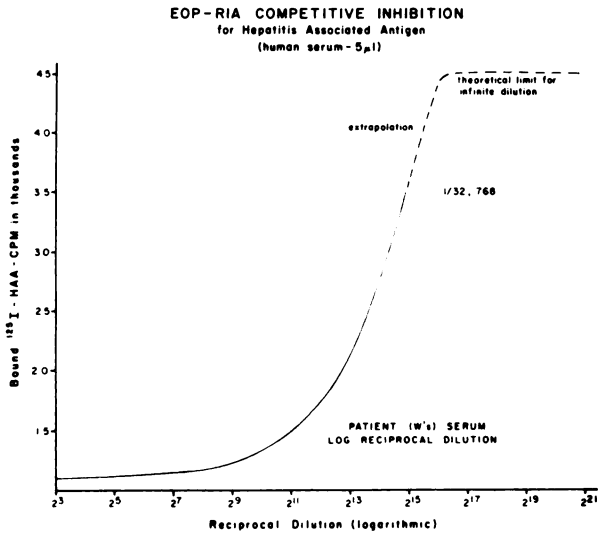

FIG. 5. EOP-RIA competitive inhibition for HAA.

is comparable to IEOP. Unlike complement fixation and RIA, the hemagglutination cannot detect the presence of antigen-antibody complex.

The primary objectives of both the single and double well techniques, described in this paper, are to retain the sensitivity of RIA while decreasing immuno-reaction time. The entire analysis from sampling to reporting by the double well technique can be completed in 2 hr, and by the single well technique in 5 hr. Recent investigations have indicated that the analysis time for the single well technique can be reduced to approximately 2–3 hr. Since the large molecules of HAA and gamma globulin diffuse slowly, compared with the other relatively small molecular constituents of serum, the single well method speeds the rate of reaction by concentrating both antigen and antibody on the perimeter of the agar well. After incubation, the bound and free antigen are then separated by electroosmophoresis. The antigen bound to antibody remains stationary while unreacted antigen (free) migrates toward the anode. Competitive inhibition is used to quantitate HAA, whereas serial dilution is used to quantitate anti-HAA.

The sensitivity of the well-type of RIA is much greater than that usually obtained by IEOP. Single well analysis detects 5 μl of anti-HAA serum (IEOP titer = 1:8) at a dilution of 4,096 in normal serum. Similarly, 5 μl of HAA positive serum (IEOP titer = 1:16) diluted in normal serum 32,768 times is easily detected without challenging the limits of detection (Fig. 5). The double well method has not been optimized for sensitivity but is able to detect HAA and anti-HAA at dilutions which are not detectable with IEOP using the same quantities of identical sera.

Different HAA-RIA procedures can be characterized by the method of separating the bound and free antigen. Walsh et al (*6*) used paper chromatoelectrophoresis, Lander et al (*8*), Aach et al (*9*), and Hollinger et al (*7*) reported double antibody separation technique. Hollinger also applied a solid phase approach. The reported incubation periods necessary for both the double antibody and solid-phase techniques verified by our experience are about 18 hr. The radioimmunoassay techniques described in this report have a sensitivity comparable to other RIA procedures but results are obtained in 2–5 hr as compared to 1–6 days for current procedures.

Recent recommendations that all bloods for transfusion be tested for HAA have spurred the search for sensitive and economical methodologies. The single and double well procedures described in this study not only offer comparable sensitivity to other radioimmunoassays but also characteristics which make automation feasible. An instrument is now under development in our laboratory that is capable of handling multiple specimens simultaneously and automates the entire procedure except for pipetting.

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