Relative Sensitivity of Gel Diffusion, Complement Fixation, and Immunoelectroosmophoresis Tests for Detection of Hepatitis-Associated Antigen and Antibody

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The immunoelectroosmophoresis (IEOP) test was compared with gel diffusion and complement fixation (CF) tests for sensitivity in detecting hepatitis-associated antigen (HAA) in the sera of hepatitis patients, for titration of HAA, and for detection of antibody to HAA. The IEOP test was found to be slightly more sensitive than either gel diffusion or CF tests for detection of antigen in the patients' sera. Titers of HAA demonstrated by IEOP were higher than those seen in gel diffusion tests but lower than CF titers. The gel diffusion test with an "enhancement" pattern was found to be more reliable than the other two procedures for detection of low levels of anti-HAA, due to the greater inhibitory effect of an antigen excess in the IEOP system and the possible masking of low levels of antibody by anticomplementary activity in the CF test system. Staining of immunoprecipitates in the IEOP test contributed little to the sensitivity of the test for detection of HAA.

It has been clearly established that human blood containing the "Australia" or hepatitis-associated antigen (HAA) may transmit the so-called "serum", or long-incubation form, of viral hepatitis to recipients (1, 5, 7, 10). In efforts to identify potentially infectious blood and blood products, attention has been focused upon the development of assay methods for HAA which possess a high degree of sensitivity for detection of the antigen and which are simple and rapid enough to permit their use for large-scale testing in blood banks.

HAA was first detected by the gel diffusion technique (2, 12) which, although simple in principle, is a relatively insensitive method for assay of the antigen. The complement fixation (CF) test was found to be more sensitive than gel diffusion for detection of HAA (4, 14–16), but the test is more complex to perform and is not generally applicable for large-scale screening. It must be conducted by highly-trained personnel, and it requires the standardization of several different test reagents and the examination of multiple dilutions of test sera. Further, the presence of low levels of antigen or antibody may be masked by the anticomplementary (AC) activity of low dilutions of test sera. Most of the procedures for gel diffusion and CF tests have the disadvantage of requiring overnight incubation before the results are obtainable.

More recently, techniques based upon immunoelectroosmophoresis (IEOP) have been described for assay of HAA and anti-HAA (6, 9, 11, 13). [In this manuscript the term immunoelectroosmophoresis (IEOP) is used synonymously with counter current electrophoresis.] This is a relatively simple procedure which can be conducted with standard electrophoresis equipment; it provides test results within an hour and is adaptable to large-scale screening. The IEOP test is also more sensitive than gel diffusion, as the antigen and antibody are drawn toward each other in the electrical field and thus are concentrated in the reaction area.

Other studies have compared the sensitivity of gel diffusion tests with that of CF (4, 14–16) or IEOP (6, 11, 13) tests for detection of HAA, but there is little information available on the comparative sensitivity of CF and IEOP tests. This report compares the sensitivity of gel diffusion, CF, and IEOP tests for detection of HAA in the sera of hepatitis patients, for titration of the antigen, and for detection of anti-HAA.
MATERIALS AND METHODS

Sera examined. Sera assayed for HAA were serial bleedings of hepatitis patients from San Francisco General Hospital. Sera examined for anti-HAA were from hemophiliacs, hepatitis patients, individuals with Down's syndrome, and normal Tongan school children who had been bred as part of a smallpox immunization study. Antiserum from hemophiliacs, a guinea pig antiserum from Courtland Laboratories, and a guinea pig antiserum from the Research Resources Branch of the National Institutes of Health were used for detection of HAA.

Gel diffusion tests. The immunodiffusion procedure developed in this laboratory for assay of HAA and anti-HAA has been described elsewhere (15). All sera were tested for antigen adjacent to a well containing a known positive antigen and for antibody adjacent to a positive antiserum; the use of such an "enhancement pattern" has been shown to increase the sensitivity of the test (15). Sera were examined without heat inactivation.

CF tests. The standard micro CF procedure of this laboratory (8, 15) was employed. Prior to examination in CF tests, sera were heated at 56 C for 30 min to inactivate native complement. Sera were examined for antigen at twofold dilutions of 1:4 through 1:2,048 against four to eight units of antibody; they were examined for antibody in block titrations against various dilutions of antigen.

IEOP tests. The protocol issued by Courtland Laboratories for counterimmunoelectrophoresis tests (Jacob C. Holper, personal communication) was followed. Sera from hepatitis patients were tested for antigen against both a human antiserum and a guinea pig antiserum from Courtland Laboratories. A Gelman electrophoresis chamber (model 51211) was employed with a Buchler 3-1008 power supply. Barbital-acetate buffer (0.04 M, pH 8.2) was used in the chamber, and the gel consisted of 1% agarose in 0.02 M barbital-acetate buffer (pH 8.2) containing 0.1% sodium azide. An 11-ml amount of melted gel was spread over a glass slide (3.25 by 4 inch; ca. 8.25 by 10.16 cm) and, after solidification of the gel and overnight storage of the plate at room temperature in a humidified chamber, wells were cut in the gel. Wells to contain antigen or test sera were 3 mm in diameter, and those to contain antiserum had a diameter of 2.5 mm; the center-to-center distance between opposing wells was 6.5 mm. Sera were examined undiluted for the presence of HAA. When sera were diluted for titration of HAA or for detection of anti-HAA, the diluent consisted of negative human serum from a single donor; this was found to be superior to a buffer diluent, as it did not give nonspecific precipitates which interfered with reading the specific reactions. After the wells were filled with the appropriate reagents, the plates were placed in the electrophoresis chamber with the antigen wells on the cathode side and the antiserum wells on the anode side. Terry-cloth wicks were used to conduct the current across the plate. Electrophoresis was conducted at 19 to 20 C by using 5.0 to 6.0 volts/cm across the plate. Tests were read at 30 and 60 min. All sera were tested without heat inactivation.

RESULTS

Comparative sensitivity of gel diffusion, CF, and IEOp tests for detection of HAA in sera of hepatitis patients. A total of 353 sera representing multiple bleedings on 74 hepatitis patients from San Francisco General Hospital were examined for HAA by gel diffusion, CF, and IEOp. Thirty of these patients showed negative reactions for HAA with all of their serum specimens in all three tests. Forty-four patients had one or more serum specimens giving a positive reaction for HAA in one or more tests.

Table 1 compares the sensitivity of the three test methods for detection of antigen in the patients' sera. Of the 353 sera examined, 182 were positive for HAA in all three tests; 158 were positive in all three tests; 3 were positive in only two tests; 10 sera were positive in one test only. Of the 10 sera which were positive in only a single test, 2 were positive by CF and 8 were positive by IEOp. Thus, the IEOp test showed a slightly greater degree of sensitivity than immunodiffusion and CF for detection of HAA in these hepatitis patients' sera.

Table 2 shows the reactions of serial serum specimens bracketing those giving discrepant results in the three tests. The sera showing discrepant results were intermediate specimens taken between an early specimen in which HAA was demonstrable by all three tests and a later one which was negative for HAA by all three tests.

With these sera, the IEOp test appeared to be slightly more sensitive than the CF test for demonstrating the persistence of antigen. However, it should be noted that sera were examined undiluted in the IEOp test and at a starting dilution of 1:4 in the CF test.

| Combination of tests | No. of sera positive | No. of sera negative |
|----------------------|----------------------|----------------------|
| All three tests      | 158                  | 182                  |
| Two tests only       |                      |                      |
| GD and CF            | 1                    | 8                    |
| GD and IEOp          | 1                    | 2                    |
| CF and IEOp          | 1                    | 0                    |
| One test only        |                      |                      |
| GD                   | 0                    | 1                    |
| CF                   | 2                    | 1                    |
| IEOp                 | 8                    | 1                    |
| Each test independently |                  |                      |
| GD                   | 160                  | 193                  |
| CF                   | 162                  | 191                  |
| IEOp                 | 168                  | 185                  |
All of the sera showing the presence of HAA in the IEOI test were positive against both the human antiserum and the guinea pig antiserum, but in several instances weaker reactions were seen with the guinea pig antiserum. Both of the antisera had CF titers of 1:32 when tested against the same antigen.

**Titers of HAA demonstrated by gel diffusion,**

**TABLE 2. Reactions of serial serum specimens on hepatitis patients who had one or more serum specimens showing discrepant results in gel diffusion (GD), complement fixation (CF), or immunoelectroosmophoresis (IEOP) tests**

| Patient | Serum (days after onset) | Results of tests for HAA<sup>a</sup> | Patient | Serum (days after onset) | Results of tests for HAA<sup>a</sup> |
|---------|-------------------------|-----------------------------------|---------|-------------------------|-----------------------------------|
|         | GD  | CF  | IEOP  | Guinea pig antiserum | Human antiserum | GD  | CF  | IEOP  | Guinea pig antiserum | Human antiserum |
| JuMa    | 14  | +   | 32<sup>b</sup> | +     | +     | MaMc | ? 1st | +   | 32   | +     |
|         | 22  | +   | 8    | 0     | 0     | ? 2nd | 0   | <4 0 | +     |
| RiBa    | 44  | 0 <4 | 0    | 0     | 0     | ? 3rd | 0   | <4 0 | +     |
|         | 6   | 2,048 | +   | +     | +     | ? 4th | 0   | <4 0 | +     |
| LaFe    | 21  | <4  | +    | 0     | 0     | ? 5th | <4 0 | 0   | +     |
| GeFr    | 36  | 0   | <4  | +     | +     | JeLe | 10  | +   | 128  | +     |
|         | 17  | 0   | 16   | +     | +     | 19   | 0   | <4 0 | +     |
| GeFr    | 27  | 128 | +    | +     | +     | LiBe | 35  | +   | 16   | +     |
| GeFr    | 30  | <4  | wk* | +     | +     | 26   | 0   | <4 0 | 0     |
| GeFr    | 10  | 128 | +    | +     | +     | 56   | 0   | <4 0 | 0     |
| LaFe    | 32  | <4  | wk* | +     | +     | 42   | 0   | <4 0 | 0     |
| LaFe    | ? >32 | 0 | <4  | 0     | 0     | 41   | 0   | <4 0 | wk* |
| LaFe    | 7   | 16  | +    | +     | +     | 70   | 0   | <4 0 | 0     |
| LaFe    | 28  | 8   | 0    | 0     | 0     | 48   | 0   | <4 0 | +     |
| LaFe    | 60  | <4  | 0    | 0     | 0     | 62   | 0   | <4 0 | 0     |

<sup>a</sup> Hepatitis-associated antigen.

<sup>b</sup> Reciprocal of CF titer.

<sup>c</sup> Weak positive reaction.

**TABLE 3. Correlation of immunoelectroosmophoresis titers with gel diffusion and complement fixation titer of hepatitis-associated antigen**

| Test            | Titer | No. of sera | Immunoelectroosmophoresis titer |
|-----------------|-------|-------------|---------------------------------|
|                 |       |             | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 |
| Gel diffusion   | 1:16  | 5           |     |     |     |     |     | 4    |     |     | 1     |
|                 | 1:8   | 6           |     |     |     | 2   | 4    |      |     |     |       |
|                 | 1:4   | 5           |     |     |     | 1   | 3    | 1    |     |     |       |
|                 | 1:2   | 7           |     |     | 2   | 3   | 2    |      |     |     |       |
|                 | 1:1   | 16          | 1   | 4   | 5   | 5   | 1    |      |     |     |       |
| Total           |       | 39          | 1   | 4   | 5   | 7   | 4    | 5    | 7    | 5     | 1     |
| Complement fixation | ≥1:2,048 | 3           |     |     |     | 2   | 1    |      |     |     |       |
|                 | 1:1,024 | 1           |     |     |     |      | 1    |      |     |     |       |
|                 | 1:512  | 5           |     |     |     | 1   | 3    | 1    |      |     |       |
|                 | 1:256  | 5           |     |     | 1   | 3   | 1    |      |     |     |       |
|                 | 1:128  | 5           |     | 1   | 1   | 2   | 1    |      |     |     |       |
|                 | 1:64   | 5           |     | 1   | 2   | 1   | 1    |      |     |     |       |
|                 | 1:32   | 5           |     | 1   | 1   | 1   | 1    |      |     |     |       |
|                 | 1:16   | 5           |     | 1   | 1   | 3   | 1    |      |     |     |       |
|                 | 1:8    | 5           |     | 1   | 2   | 2    |      |     |     |     |       |
| Total           |       | 39          | 1   | 4   | 5   | 7   | 4    | 5    | 7    | 5     | 1     |
| Patient group         | Patient      | GD | CF (titer) | Anti-HAA detectable by | IEOP | Courtland antigen | Antigen BeZe |
|----------------------|--------------|----|------------|------------------------|------|-------------------|--------------|
|                      |              |    |            |                        |      | 1:10              | 1:20         |
| Hemophiliacs         |              |    |            |                        |      | 1:10              | 1:20         |
| 1. KeKi 8896         | +            | 1:32 | +          | +                      | +    |                   |              |
| 2. Ebe. NIH          | +            | 1:32 | +          | +                      | +    |                   |              |
| 3. Kelly             | +            | 1:20 | +          | +                      | +    |                   |              |
| 4. DoPe 8712         | +            | 1:16 | +          | 0                      | +    |                   |              |
| 5. DaGo 10227        | +            | 1:8  | +          | +                      | +    |                   |              |
| 6. RiCl 8707         | +            | 1:8  | +          | +                      | +    |                   |              |
| 7. DoFl 5428         | +            | 1:8  | +          | +                      | +    |                   |              |
| 8. LaHa 5758         | +            | 1:4  | +          | +                      | +    |                   |              |
| 9. HeWo 1712         | +            | 1:4  | +          | 0                      | +    |                   |              |
| 10. RiPo 10452       | +            | 1:4  | +          | 0                      | +    |                   |              |
|                     | RiPo 5767    | +   | 1:2        | 0                      | 0    |                   | +            |
|                     | JaBa 2083    | +   | 1:4        | 0                      | 0    |                   | +            |
|                     | JaBa 4521    | +   | 1:2        | 0                      | 0    |                   | +            |
|                     | RiCo 1427    | +   | 1:2        | 0                      | 0    |                   | +            |
|                     | JaCa 4530    | +   | 1:2        | 0                      | 0    |                   | +            |
|                     | HaWo 1713    | +   | 1:4        | 0                      | 0    |                   | +            |
|                     | JaSi         | +<1:4 | 0         | 0                      | +    |                   | +            |
|                     | through 24   | 0   | <1:4       | 0                      | 0    |                   | +            |
| Down's syndrome      |              |    |            |                        |      |                   |              |
| Hepatitis patients   |              |    |            |                        |      |                   |              |
| 25. DaWi S-77        | +            | <1:4 | 0          | 0                      | 0    |                   | +            |
| 26. LaWr 7306        | +            | 1:4  | 0          | 0                      | 0    |                   | +            |
|                     | ElRu 623     | +<1:4 | 0         | 0                      | 0    |                   | +            |
|                     | JuSo 627     | +   | 1:8        | 0                      | 0    |                   | +            |
|                     | PaBe 748     | +   | 1:4        | 0                      | 0    |                   | +            |
|                     | SuJo 225     | +<1:4 | 0         | 0                      | +    |                   | +            |
|                     | KaKe 237     | +   | 1:4        | 0                      | 0    |                   | +            |
| Tongan school        |              |    |            |                        |      |                   |              |
| children             |              |    |            |                        |      |                   |              |
| 32. AI-23-30         | +            | 1:8  | 0          | 0                      | 0    |                   | +            |
| 33. AI1-09-0         | +            | 1:4  | 0          | 0                      | 0    |                   | +            |
| 34. 2AI-117-0        | +            | 1:4  | 0          | 0                      | 0    |                   | +            |
| 35. DI-28-30         | +            | 1:4  | 0          | 0                      | 0    |                   | +            |
| 36. 2DI-04-21        | +            | 1:4  | 0          | 0                      | 0    |                   | +            |
| 37. CI-08-00         | +            | 1:2  | 0          | 0                      | 0    |                   | +            |
| 38. 2BI-14-21        | +<1:8         | +  | <1:8       | 0                      | 0    |                   | +            |
| 39. 2DI-28-21        | +<1:8         | +  | 1:4        | 0                      | 0    |                   | +            |
| 40. 2BII-14-21       | +            | 1:4  | 0          | 0                      | 0    |                   | +            |

* a Serum gave a positive reaction for anti-HAA only when tested in an enhancement pattern, i.e., adjacent to a positive antiserum.
* b Anticomplementary at lower dilutions.

**CF, and IEOP tests.** It has been reported that titers of HAA obtained in IEOP tests are markedly higher than those obtained on the same sera in gel diffusion tests (6, 13), but little information is available on comparative titers in CF and IEOP tests. Sera with a range of different CF titers for HAA were titrated in IEOP and gel diffusion tests by using the same human antiserum which had been employed for CF tests. Results are shown in Table 3. HAA titers obtained in IEOP tests were generally 4- to 16-fold higher than those seen for the same sera in gel diffusion tests, but IEOP titers were lower than CF titers. Only 3 of the 39 sera tested showed the same titers by IEOP and by CF.

**Comparative sensitivity of gel diffusion, CF, and IEOP tests for detection of anti-HAA.** Table 4 compares the sensitivity of the three test procedures for detection of anti-HAA in the sera of hemophiliacs, hepatitis patients, and presumably normal Tongan school children. Antibody assays were conducted against a 1:10 dilution, and in
some instances a 1:20 dilution, of an antigen from Courtland Laboratories and against various dilutions of an antigen (BeZe) from this laboratory. The Courtland antigen had a titer of 1:128 by IEOp, and antigen BeZe had a titer of 1:256. Human sera with anti-HAA CF titers of 1:8 or higher gave a positive IEOp reaction with 1:10 and higher dilutions of both antigens and, in most instances, even with undiluted BeZe antigen. However, with lower titered antisera, a marked inhibitory effect of excess antigen was noted; sera which gave positive reactions for anti-HAA in gel diffusion tests only when an enhancement pattern was used generally gave a positive IEOp reaction only with a 1:40 or 1:80 dilution of antigen. Thus, IEOp tests for anti-HAA with a single, low dilution of antigen were less reliable than gel diffusion tests with an enhancement pattern (testing sera adjacent to a positive antiserum). The detection of low levels of anti-HAA by IEOp required the use of relatively high dilutions of antigen to overcome the inhibitory effect of an antigen excess.

Patients 16 through 24 shown in Table 4 were hemophiliacs who had been multiply transfused, but whose sera showed no evidence of anti-HAA in gel diffusion or CF tests; the sera also failed to show anti-HAA in IEOp tests, even when tested against dilute antigen.

Effect of staining on the sensitivity of the IEOp test. To determine the extent to which staining the precipitates might enhance the sensitivity of the IEOp test, 117 human sera, 74 of them positive for HAA by CF and 43 negative by CF, were run in IEOp tests against three different antisera, and readings were made on coded preparations before and after staining. The acid fuchsin staining method described by Campbell et al. (3) was employed. Table 5 shows that, with tests performed against the Riv. human antiserum and against the NIH guinea pig antiserum, staining demonstrated two or three additional positive reactions not seen in unstained preparations with the 74 sera that were positive by CF. In tests against human antiserum, KeKi staining demonstrated four additional positive reactions with the 74 sera, but it also gave four apparent false-positive reactions in tests with sera negative by CF and by IEOp with the other antisera. Thus, the sensitivity of the IEOp test was not markedly increased by staining the immunoprecipitates.

| Table 5. Effect of staining on the sensitivity of immunoelectroosmophoresis (IEOp) tests for hepatitis-associated antigen (HAA) |
|---|---|---|---|---|---|
| Reactions of sera in CF test for HAA | No. of sera | No. of positive IEOp reactions vs. antiserum |
| | | Human (Riv.) | NIH guinea pig | Human (KeKi) |
| | | Unst<sup>a</sup> | Stained<sup>b</sup> | Unst | Stained | Unst | Stained |
| Positive (titer ≥1:4) | 74 | 71 | 74 | 72 | 74 | 70 | 74 |
| Negative (titer <1:4) | 43 | 0 | 0 | 0 | 0 | 4 |

<sup>a</sup> Unstained preparations.
<sup>b</sup> Stained with acid fuchsin.

Discussion

In this study, the IEOp test was found to be as sensitive as the CF test for detection of HAA in the sera of hepatitis patients, and it was slightly more sensitive in detecting antigen in a few convalescent-phase sera in which antigen titers had decreased to a level of <1:4 in CF tests. The ability of the IEOp test to detect antigen in these few sera negative by CF might have been due to the fact that sera were examined undiluted in IEOp and at a starting dilution of 1:4 in CF tests.

Titers of HAA demonstrable by IEOp were lower than those detected by CF, usually on the order of fourfold lower. This may be attributable to the fact that a positive reaction in IEOp requires the formation of sufficient antigen-antibody precipitate to give a visible reaction, whereas the CF test detects smaller, soluble antigen-antibody complexes. However, in actual practice, it is the detection of HAA in blood products which is important, not the demonstration of maximum titers of antigen. The use of undiluted test serum in the IEOp test permits testing for HAA at high serum concentrations at which AC activity might prevent the detection of antigen in CF tests.

The simplicity of the IEOp test, the speed with which results can be obtained, and its relatively high degree of sensitivity in detecting HAA would tend to make it the procedure of choice for use in large-scale screening of blood products.

Although it is less sensitive, the gel diffusion test can be a valuable adjunct to the IEOp test. Sera giving a positive reaction by IEOp can be
tested by immunodiffusion to confirm the specificity of the precipitation reaction as being due to HAA. If an “enhancement” pattern is employed in which sera are tested against antibody in the central well and adjacent to a known positive antigen in peripheral wells, the sensitivity of the immunodiffusion test for detection of HAA is markedly increased (15); the identity of positive reactions as being due to HAA is confirmed; and test sera are assayed for antigen and antibody simultaneously.

The IEOP test is less useful than the gel diffusion test for detection of anti-HAA because of the greater inhibitory effect of an antigen excess in the IEOP system. Testing sera by IEOP for antibody simultaneously with the test for antigen by using an antigen-containing well on the cathode side of the test serum well is not a reliable practice since a range of antigen dilutions may be required to permit detection of low levels of anti-HAA. In the present study, 17 of the 31 sera containing anti-HAA gave a negative IEOP reaction against the 1:10 dilution of antigen recommended for detection of anti-HAA in the Courtland Laboratories protocol. Of the three test systems, the gel diffusion test employing an enhancement pattern would appear to be most suitable for detection of anti-HAA from the standpoint of simplicity and sensitivity. The presence of anti-HAA in the serum of an individual is considered to be indicative of a past hepatitis infection, but it has not yet been determined whether blood products containing anti-HAA should be rejected; if such a practice is established, it will become important to use a rapid test for anti-HAA as well as for HAA, and it may become necessary to screen bloods for anti-HAA by IEOP against a range of antigen dilutions.

Staining of the immunoprecipitates in IEOP tests results in little increase of sensitivity and would not appear to be warranted in light of the lengthened time required for obtaining test results and the increased risk of infection involved in performing the staining procedure.