Evaluation of Passive Hemagglutination, Solid-Phase Radioimmunoassay, and Immunoelectroosmophoresis for the Detection of Hepatitis B Antigen

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Sensitivity and specificity of passive hemagglutination (RCA), solid phase radioimmunoassay (RIA), and immunoelectroosmophoresis (IEOP) were compared under experimental and clinical conditions. In dilution experiments with sera containing hepatitis B antigen (HB Ag) of known subtypes, the sensitivity for an ad subtype serum was RIA (1), RCA (1/2), IEOP (1/256) and for an ay subtype serum RCA (1), RIA (1/8), IEOP (1/128). An evaluation of the National Institutes of Health, Division of Biologics Standards test panel number 2 demonstrated HB Ag in 34 of 60 samples by RIA, in 33 by RCA, and in 25 by IEOP. HB Ag was detected in 57.5% of 200 outpatients with a tentative diagnosis of hepatitis by RIA, in 54% by RCA, and in 42.5% by IEOP. In 1,661 volunteer blood donors, 13 (0.78%) were "positive" for HB Ag by RIA, 11 (0.66%) by RCA, and 3 (0.18%) by IEOP. However, absorption experiments indicated that at least six of the above RIA positive and five of the RCA positive sera exhibited nonspecific positive reactions.

Testing of blood donors for hepatitis B antigen (HB Ag) is necessary because of the close association between HB Ag in blood donor sera and the development of post-transfusion hepatitis (5, 6, 11). It has been estimated that agar gel diffusion, immunoelectroosmophoresis (IEOP), and complement fixation methods detect only 20 to 40% of the hepatitis-transmitting blood units (1, 4, 13). This could be due to the insufficient sensitivity of current test methods for HB Ag (3). An evaluation of agar gel diffusion, IEOP, and complement fixation has shown that none of the three methods was capable of consistently detecting HB Ag when it was present in relatively low concentrations in human serum (8). Therefore, radioimmunoassays (4, 9, 18) and passive hemagglutination inhibition assays (17), which are among the most sensitive means of detecting minute amounts of antigen, have been developed. The aim of this work is to compare the sensitivity and the specificity of a passive hemagglutina-

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tion test (RCA) and a solid-phase radioimmunoassay (RIA) with IEOP, the method most used currently.

MATERIALS AND METHODS

Reference sera. The HB Ag reference panel no. 2 was obtained from the National Institutes of Health, Division of Biologics Standards (NIH-DBS). Sera containing HB Ag of the ad and ay subtype were kindly supplied by C. M. Ling (Abbott Laboratories).

Blood donor sera. Between 23 October and 5 December 1972, serum samples from 1,661 volunteer blood donors were collected. Within 48 h after they had been obtained, 1,070 sera were tested without freezing. The other sera were stored at −20 C until tested.

Outpatient sera. During a 2-year period (1969–1971), sera were collected in a city hospital for communicable diseases from 200 outpatients who were diagnosed clinically as having acute hepatitis and whose serum glutamic-oxalacetic transaminase values exceeded 100 U/ml (14). The sera were stored at −20 C until used. The outpatients were mainly residents of a densely populated inner city area which has a low socioeconomic level. The first serum drawn after onset of the disease (usually within 3 weeks) was tested.
Immunoelectrophoresis. IEOP was done with the "Hapindex-60 Test" in disposable slide-cassettes (Ortho-Diagnostics). Hepatitis B antibodies (HB Ab) from rabbits were used.

Radioimmunoassay. For a solid-phase RIA, the "Austec" test was used (Abbott Laboratories). A sample (0.1 ml) of a test serum was added to polystyrene tubes coated with guinea pig HB Ab. After 16 h of incubation at room temperature, tubes were washed five times with 2-ml of Tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (0.005 M, pH 7.1, 0.1% sodium azide), and 0.1 ml of 125I-labeled guinea pig HB Ab was added. After incubation for 1.5 h at room temperature and a second wash with Tris buffer, the tubes were counted in a gamma counter and results were calculated as suggested by the manufacturer (positive: \( \geq 2.1 \times \text{mean of seven simultaneous tests with one standardized negative control} \).

Passive hemagglutination for HB Ag. RCA was performed with the "Austect" test (Abbott Laboratories; evaluation test, unlicensed investigational reagents). In this test, agglutination of formalized human erythrocytes (7), sensitized with guinea pig HB Ab, indicates the presence of HB Ag. Screening and titrations of test sera were done in microtiter plates (Linbro, mold 4) with microtiter equipment (Cook Engineering Co.). For screening, one drop (0.025 ml) of an 1:8 dilution of the test serum in dilution buffer (0.08 M phosphate solution with gelatin) was mixed with the same volume of sensitized or nonsensitized erythrocytes. Plates were read after a 2-h incubation at room temperature, and if hemagglutination was present, a titration of the serum was made. Results were considered as positive when the agglutination titer with sensitized cells exceeded the titer with the control cells fourfold or more. Sera which were bacterially contaminated were spun for 10 min at 2,000 rpm in an International centrifuge to diminish nonspecific agglutination.

**Absorption experiments.** Sera positive for HB Ag in RCA were mixed in a 1:1 ratio with different adsorbents and incubated overnight at 4°C, then diluted 1:4 (final 1:8) and retested after centrifugation at 2,000 rpm for 10 min. Sera positive in IEOP or RIA, or both, were mixed and incubated in the same manner, but were retested without further dilution and centrifugation.

**Results**

Sensitivity of tests in dilution experiments. Twofold dilutions of sera containing the ad or ay subtype of HB Ag were made and tested by IEOP, RCA, and RIA (Table 1). RIA was the most sensitive method for the ad subtype serum whereas RCA exhibited one-half and IEOP only 1/256 the sensitivity of RIA. Similar results were also found by Matsuda and co-workers (10) by using a standard mixture of HB Ag-positive human serum. For the ay subtype serum the range was RCA (1), IEOP (1/8), IEOP (1/128). RIA was less sensitive for the detection of the ay subtype in this particular serum. However, there may be variation in sensitivity for subtypes from serum to serum. In general, under these experimental conditions, RIA and RCA exhibited roughly the same sensitivity and were clearly superior to IEOP in detection of HB Ag.

**Evaluation of an HB Ag reference panel.**

| Table 1. Subtypes of hepatitis B antigen detected by IEOP, RCA, and RIA |
|-----------------------|-------------------|-------------------|-------------------|
| Serum dilution        | ad-type           | ay-type           |
| (\(-\log 2\))        | IEOP (cpm)        | IEOP (cpm)        | IEOP (cpm)        |
| 1                     | Prozone           | Prozone           | 1/256             |
| 2                     | ≥2,048            | ≥2,048            | 1/2               |
| 3                     | ≥2,048            | 15,341            | 1/128             |
| 4                     | 15,984            | +                 | 1                 |
| 5                     | 15,336            | +                 | 1                 |
| 6                     | 15,086            | +                 | 1                 |
| 7                     | 15,422            | +                 | 1                 |
| 8                     | 13,391            | +                 | 1                 |
| 9                     | 12,548            | +                 | 1                 |
| 10                    | 10,619            | +                 | 1                 |
| 11                    | 9,418             | +                 | 1                 |
| 12                    | 2,048             | +                 | 1                 |
| 13                    | 2,813             | +                 | 1                 |
| 14                    | 8,325             | -                 | 1                 |
| 15                    | 4,377             | -                 | 1                 |

* Each -\(\log 2\) dilution was considered as an undiluted test sample and was titrated in a standard RCA test. The titer is given as the reciprocal of the last test dilution giving positive results.

* Positive \(\geq 1,170\) counts per min (see text).
The NIH-DBS HB Ag reference panel no. 2 offered another comparison of the sensitivity of the different tests under control experimental conditions (Table 2). According to NIH-DBS, this panel contained specimens with various concentrations of HB Ag, ranging from samples easily recognized by agar gel diffusion to those which borderline positive results even in the most sensitive method used (RIA).

In our laboratory, RIA was able to detect all 34 clear positive samples, but missed the six borderline positive sera. RCA showed nearly the same sensitivity as RIA in detecting 33 of the 34 clear positive samples. One positive sample of the ad type, expected to be detected by more sensitive methods than IEO and complement fixation, was not found. IEOP in our hands detected only 25 positive samples. The greater sensitivity of RIA and RCA in detecting HB Ag in these sera was obvious.

Testing of blood donors and outpatients. To determine whether additional positive sera would be detected by RIA and RCA on a clinical level, a population of outpatients with a tentative diagnosis of hepatitis and a volunteer blood donor population were tested for HB Ag (Table 3). The outpatients were presumed to have a high incidence, and the volunteer blood donors a low incidence, of HB Ag. From 200 sera of outpatients, 115 were positive for HB Ag by RIA, 108 by RCA, and only 85 by IEO. Again, RIA and RCA appear much more sensitive than IEOP in detection of HB Ag.

From 1,661 sera of volunteer blood donors from the Chicago metropolitan area, three showed positive results in IEOP and were also positive in RIA and RCA. One serum was found to be positive in RIA and RCA only, but negative in IEOP. Seven sera were positive only in RCA with relatively low titers (1:32 to 1:512), and nine other sera gave positive results in RIA only.

No significant difference was exhibited by the three test methods between fresh and frozen sera. Retesting of fresh sera after freezing and several weeks storage did not alter previous positive test results.

Absorption experiments. The surprising high and discordant positive results in the donor population by RIA and RCA were further tested for specificity. Alter and co-workers (2) have reported false positive tests for HB Ag in blood donors caused by antibodies to ruminant serum proteins if either ruminant thrombin was present in the screening reagents or if HB Ab produced in ruminants was used. Because the HB Ab in both RIA and RCA tests were derived from guinea pigs, false positive results have been reported due to antibody against guinea pig serum (12, 15). Therefore, absorption experiments (Table 4) were done with phosphate-buffered saline (PBS), pH 7.2, human serum negative for HB Ab by passive hemagglutination (17), rabbit serum, guinea pig serum (unheated and inactivated for 30 min at 56 C), and a calf thrombin solution (100 NIH U/ml in PBS; Parke, Davis & Co.). From the sera positive in RCA only, all five which were available for retesting were negative in RCA after absorption with guinea pig serum or calf thrombin. However, these same sera remained positive in RCA after absorption with PBS, human serum, or rabbit serum. From eight available sera, posi-

| Test method for HB Ag | Evaluation |
|-----------------------|------------|
|                       | NIH-DBS    | Chicago |
| Immunoelectroosmophoresis positive samples | 28 (2)* | 25 |
| Radioimmunoassay positive samples | 34 (6) | 34 |
| Passive hemagglutination positive samples | NT* | 33 |
| Samples non-reactive by all tests | 20 | 26a |

*The panel contained 60 samples; no. 252 was missing. Therefore, the NIH-DBS results have no. 252 subtracted to allow a more direct comparison.

*Number in parentheses indicates number of sera giving borderline results in addition to the clear positive samples as defined by NIH-DBS.

*NT, Not tested.

*These include the 20 nonreactive samples of the NIH-DBS evaluation and the six which NIH-DBS called borderline in radioimmunoassay.

| Group | No. of sera positive | Positive results in |
|-------|----------------------|---------------------|
|       | IEO | RCA | RIA |
| 200 outpatients with tentative diagnosis of hepatitis | | |
| 85 | + | + | + |
| 23 | - | + | + |
| 7 | - | - | + |
| 115 | (57.5%) | 108 | (54%) |
| 1,661 volunteer blood donors | | |
| 3 | + | + | + |
| 1 | - | + | + |
| 7 | - | + | - |
| 9 | 20 | 3 | 11 | 13 |
| 25% | (1.2%) | (0.18%) | (0.66%) | (0.78%) |
TABLE 4. Absorption studies with sera positive for HB Ag from 1,661 volunteer blood donors

| Conditions      | Test | No. of sera positive for HB Ag in |
|-----------------|------|----------------------------------|
|                 | IEOP + RIA + RCA | RIA + RCA | RCA | RIA |
| Before absorption | 3* (3) | 1 (1) | 5 (7) | 8 (9) |
| Absorbed with | Human serum* | RIA | 3 | 1 | ANA* | 6* |
| Rabbit serum   | RIA | 3 | 1 | ANA |
| Guinea pig serum/ | RIA | 3 | 1 | ANA |
| Calf thrombin* | RIA | 3 | 1 | ANA |
| Rabbit HB Ab*  | RIA | 0 | - | ANA |
| RCA            | 0 | - | 5 | ANA |

* Number of sera available for absorption; the number in parentheses indicates the total sera originally found positive (Table 3).
* Negative for HB Ag and HB Ab.
* ANA, Absorption not appropriate.
* Two sera became negative after a 1:2 dilution with PBS and therefore could not be tested.
* No serum available.
* Inactivated (56 C, 30 min) and unheated serum gave the same results.
* Calf thrombin (Parke, Davis & Co.), 100 NIH U/ml in PBS.
* HB Ab, Hepatitis B antibody.

tive in RIA alone, absorption of nonspecific activity with guinea pig serum (but not with calf thrombin) could be shown in six sera. The other two sera gave negative results in the RIA test when diluted 1:2 with PBS or human serum, and absorption of nonspecific reactivity could therefore not be demonstrated in these two sera. To show that only nonspecific agglutinating factors were removed by the absorption techniques, the following experiment was performed. The three sera which had been positive in IEOP, RIA, and RCA were diluted in PBS to give results in RIA or RCA similar to those obtained with sera reacting only in RIA or RCA (comparable number of counts per minute or agglutination titer). These serum dilutions and, in addition, the undiluted serum which had been positive in both RIA and RCA were then absorbed and tested as described above. None of the sera lost its reactivity by the absorption procedure.

In addition, lack of absorption of the agglutinating factor from sera positive only in RIA or RCA with specific rabbit HB Ab further proved that these reactions were nonspecific (Table 4) since the reactivity of sera positive in all three tests could be completely abolished by absorption with HB Ab. Therefore, in this volunteer blood donor population, only one HB Ag-containing serum (positive in both RIA and RCA) was detected in addition to the three IEOP positive sera. Sera of 23 outpatients positive in RIA and RCA but negative in IEOP remained positive when retested by RIA and RCA after absorption with guinea pig serum. Insufficient amounts of sera prevented absorption studies on the seven outpatients who were positive for HB Ag by RIA only.

**DISCUSSION**

RIA and RCA were of nearly equal sensitivity and clearly superior to IEOP in testing of serum dilutions containing HB Ag of known subtype, an NIH-DBS reference panel, and outpatients with tentative diagnosis of hepatitis. But the number of additional carriers detected in a blood donor population was not as much as expected from the comparison of the sensitivity of these tests in dilution experiments. However it has been emphasized that most carriers in donor populations tend to have levels of HB Ag that are easily detected by IEOP (16). Our results support this suggestion.

Data obtained from the absorption experiments indicate that nonspecific positive results occur relatively seldom in both RIA and RCA but are of importance in testing blood donor populations with low incidence of HB Ag. Different mechanisms seem to be responsible for the nonspecific positive results in RCA or RIA.
All samples which became negative after absorption were originally positive in only one of the two tests in spite of the nearly equal sensitivity of RIA and RCA. The nonspecific reactive sera positive in RCA could be absorbed with both guinea pig sera and calf thrombin, whereas only guinea pig sera were effective in absorbing nonspecific reactivity from sera positive in RIA only. Because fresh and heat-inactivated guinea pig sera showed the same results, the complement system cannot be responsible for the false positive results. The nature of the nonspecific positive results could not be evaluated completely because only small amounts of serum were available for the absorption experiments.

The RCA test has sensitivity comparable to RIA, it is easy to perform with simple equipment, the total time for completion of a test is short (3 h), nonspecific positive results seem not to occur too frequently and can be controlled by simple absorption of the test sera; this test therefore warrants further study and evaluation.

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ADDENDUM IN PROOF

Current RIA and RCA tests (Abbott Laboratories) include modifications of the tests used here which minimize the number of false positive results due to the presence of antibody which reacts with guinea pig serum.

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