An Enzyme-Linked Immunosorbent Assay (ELISA) for Measurement of Heterophile Antibody

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An enzyme-linked immunosorbent assay (ELISA) test has been developed for measurement of heterophile antibody. The microtiter test utilizes a bovine erythrocyte monolayer as antigen and anti-human IgM antiserum conjugated with horseradish peroxidase to measure the degree of binding of the heterophile antibody in the test serum with the erythrocytes. A single serum dilution yields quantitative results when read in a spectrophotometer. The ELISA test showed a sensitivity comparable with the immune adherence hemagglutination assay (IAHA) and other heterophile tests, good reproducibility, and high specificity.

The enzyme-linked immunosorbent assay (ELISA) technique is being widely used in diagnostic laboratories as a sensitive and rapid method for measurement of various antibodies, for detecting antigen, and for other immunoassays [1]. The increasing familiarity of technicians with ELISA methods and the increasing availability of ELISA readers capable of accurate quantitation of the color developed by the enzyme substrate, even in microtiter plates, suggested that a simple heterophile test based on these principles and using a single dilution of serum might serve a useful purpose.

In the current study a modified enzyme-linked immunosorbent assay (ELISA) for the Paul-Bunnell (PB) antibody of infectious mononucleosis has been developed. The assay utilizes as antigen a bovine erythrocyte monolayer in a microtiter well, and horseradish peroxidase (HRP) conjugated anti-human (IgM) serum to measure the degree of IgM binding. Quantitation was achieved spectrophotometrically by measuring the colored substrate products at one single serum dilution. A description of this assay and of its sensitivity and specificity is presented in this paper.

MATERIALS AND METHODS

Serum Samples

Serum and/or plasma samples from 22 cases of infectious mononucleosis in Yale University students, whose disease fulfilled clinical, hematological, and serological criteria [2] and specimens from healthy adults were tested. Control sera came from...
ten healthy adults of the same age and from 17 young adults with high antibody titers against other viral infections. The serum samples had been stored at $-20^\circ$C for variable periods of time. Some sera were also tested prior to freezing.

Both sera and plasma were heat-inactivated at $56^\circ$C for 30 minutes prior to testing. The results of other heterophile antibody tests and of EBV/IgA antibody measurements on these same infectious mononucleosis sera have recently been reported elsewhere [3,4].

**Heterophile Tests**

The absorbed heterophile agglutination tests using either sheep or horse red cells were carried out on microtiter plates utilizing stored frozen sera as previously described from this laboratory [3]. The Immune Adherence Hemagglutination Assay (IAHA) was modified from the method of Lennette et al. [5] by using samples frozen at $-20^\circ$C. Our comparative results with this sensitive test have recently been published [4].

**ELISA for Heterophile Antibody**

(1) **Plates** In all tests flat-bottom microtiter plates with 96 wells were used. These were either tissue culture (TC grade) plates purchased from Linbro (New Haven, CT) or from Cooke Engineering (Alexandria, VA) or ELISA “grade” polystyrene plates purchased from Dynatech Co. (South Windham, ME). In most experiments the former were employed. All gave satisfactory results but the TC grade plates were used in most experiments.

(2) **Reagents** Bovine Rbc's in Alsever's solution were obtained from Flow Laboratories (McLean, VA). No difference in batches of Rbc was seen in or in storage at $4^\circ$C up to at least five weeks. The red cells were washed three times in phosphate buffered saline (PBS) before use. This PBS buffer, used throughout the entire procedure, contained (per liter) 8 gm NaCl, 0.2 gm KCl, 0.2 gm KH$_2$PO$_4$, 2.9 gm NaH$_2$PO$_4$•$7$H$_2$O. Premixed salts were purchased from Gibco (Grand Island, NY). Poly-L-Lysine (PLL) hydrobromide type 1-B and horseradish peroxidase (HRP), Type VI 5000 $\mu$/20 mg were purchased from Sigma Chemicals (St. Louis, MO). O-phenylene-diamine (OPD), practical grade (P-1700) was obtained from Eastman Kodak Co. (Rochester, NY). A stock solution of OPD was prepared by dissolving 100 mg OPD in 10 ml absolute methanol and maintained at $-20^\circ$C in darkness. Working solutions contain 1 ml stock, 0.1 ml 3 percent H$_2$O$_2$ in 100 ml water solution and may be stored in the dark at $4^\circ$C for up to two weeks satisfactorily if no color change occurs. HRP-conjugated goat anti-human IgM, IgG fraction (Cappel Laboratories, Cochranville, PA) and HRP-goat anti-human IgG and HRP-goat anti-human IgA were obtained from Litton Bionetics (Kensington, MD). Trypsin solution in Ca$^{++}$/Mg$^{++}$ free Hanks was purchased from Flow Laboratories (McLean, VA).

(3) **Preliminary Evaluation of Test Conditions** Variables which were tested included: different microtiter plates, dose-response curves at different serum and conjugate concentrations, various incubation times and various temperatures of incubation. The "standard procedure" described below is based on the optimal conditions from these experiments.

(4) **Standard Procedure**

(a) Monolayer preparation: 50 $\mu$l of poly-L-Lysine (PLL) (100 $\mu$g/ml in distilled water containing 50 percent ethyl alcohol) was placed in each well of a flat-bottom microtiter plate with a Hamilton syringe and allowed to stand at room temperature
for 30 minutes before decanting and rinsing once with 100 \( \mu l \) PBS. 50 \( \mu l \) of 10 percent suspension of bovine Rbc in PBS was added to each well and the plate was placed on a flat surface for 40 minutes before being washed three times with PBS to obtain a uniform monolayer [6].

(b) Incubation with serum sample: 50 \( \mu l \) of serum at a 1:20 dilution was added to each well, usually in triplicate. The plate was incubated at 22°C to 37°C for two hours.

(c) Incubation with conjugate: The plate was washed once with PBS, lysed by adding 100 \( \mu l \) cold hypotonic PBS @ 20 ideal milliosmolar (imOsm) for 30 seconds and washed three more times with PBS. Similar results were obtained whether the lysis of red cells was performed before or after the addition of serum. 50 \( \mu l \) each of an appropriate dilution of conjugate was added, incubated at room temperature for 50 minutes, then the plate was washed five times with PBS. Each wash was done by filling the well with 250 \( \mu l \) of PBS, holding for one minute, then decanting. Tween buffer was not used.

(d) Adding substrate: 200 \( \mu l \) of OPD was added to each well and kept in the dark for 20 minutes before adding 50 \( \mu l \)/well of 8 N H\(_2\)SO\(_4\) by means of a transfer plate to stop the reaction. A further five minutes was required to stabilize the color before reading.

(e) Readings: The plate was read with the Titretek Multi-Skan eight-channel microtiter plate spectrophotometer (Flow Laboratories, McLean, VA), using a 492 nm filter. The first column in each plate contained only ghost monolayer cells and substrate: its reading was used as the background value which was automatically subtracted by the machine from all subsequent readings on the printout.

**Notes on the Test Conditions**

The plate used should have a good monolayer with few pinholes. When ethanol was used in PLL solution, both tissue culture grade and ELISA plates yielded satisfactory results. In most experiments we used TC plates, as they formed better monolayers more consistently than other types of plates tested. Prior absorption of the test sera with guinea pig kidney did not alter the reading, whereas prior absorption with beef Rbc completely abolished it. These are the characteristics of the heterophile antibody of infectious mononucleosis. Dose response curves of either purified horseradish peroxidase or HRP-anti-IgM conjugate added directly to the substrate gave good correlation between the spectrophotometer reading and the amount of conjugate present.

**RESULTS**

**Preliminary Determinations**

The results of the ELISA test on twelve fresh serum samples from cases of acute infectious mononucleosis and on ten normal control subjects are shown in Fig. 1. The ELISA value of the control sera averaged 0.15 (range, 0.1 to 0.2); no value was over 0.25, which was taken as the upper limit of normal. A value of 0.25 to 0.35 was taken as marginal, and values over 0.35 as positive for fresh serum samples. With one exception the IM sera all gave values of 0.35 or higher (average, 1.05).

**Effect of Storage**

The effect of storage of sera at 4°C for a week is shown in Table 1. About a 40 percent drop in ELISA readings occurred in either fresh or previously frozen sera
stored in this way. Fresh sera also gave values about twice as high as sera stored at −20°C for variable periods. Despite this, a value of 0.25 or higher as elevated proved a useful guideline for frozen sera as well as for fresh sera.

**Sensitivity**

The ability of the ELISA test to measure heterophile antibody in infectious mononucleosis cases was determined in 16 sera containing different levels of heterophile antibody as determined by two sensitive assays. Values for the ELISA test, even on those previously frozen sera, were above 0.25 for all sera at a positive level of ≥1:40 by the IAHA and/or horse Rbc test (Table 2). In addition, the ELISA value was elevated to 0.393 in one serum with an IAHA titer of only 1:20.

**Specificity**

Seventeen previously frozen sera with elevated antibody titers (≥1:160) against other herpes, rubella, or measles viruses but which were heterophile-negative by the horse cell tests gave ELISA readings below 0.25, with two exceptions. One of these was from a person with a rubella H.I. titer of 1:320 and gave ELISA readings of 0.423 in triplicate readings. This came from a case of Hodgkin's disease and had an EBV-VCA titer of 1:40; other antibody titers were normal (see Fig. 2).

**TABLE 1**

| Status of Sera at Start | Number of Sera | H.A. Titer Range | Initial Reading | After 1 Week at 4°C | % Decrease |
|-------------------------|----------------|------------------|-----------------|---------------------|------------|
| Fresh                   | 3              | High             | 1.414           | 0.786               | 44.4       |
| Frozen*                 | 22             | High             | 0.733           | 0.448               | 38.8       |

*At −20°C for variable periods
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TABLE 2
Correlation of ELISA Readings with High, Medium, Low and Negative Heterophile Titers as Determined by the IAHA and Horse Rbc Agglutination Tests

| Serum Number | Heterophile Group | IAHA Titer | Horse Rbc Titer | ELISA Reading at 1:20 Serum Dilution |
|--------------|------------------|------------|-----------------|-------------------------------------|
| 1            | High Titer       | 10240      | 10240           | 0.727                               |
| 2            |                  | 5120       | 2560            | 0.558                               |
| 3            |                  | 10240      | 5120            | 1.028                               |
| 4            |                  | 10240      | 10240           | 1.253                               |
| 5            |                  | 10240      | 5120            | 0.684                               |
| GMT*         |                  | 8914       | 5881            | 0.85** Av.                          |
| 6            | Medium Titer     | 320        | 640             | 0.475                               |
| 7            |                  | 640        | 320             | 0.461                               |
| 8            |                  | 2560       | 640             | 0.540                               |
| 9            |                  | 1280       | 1280            | 0.531                               |
| 10           |                  | 2560       | 1280            | 0.448                               |
| GMT          |                  | 1114       | 735             | 0.491 Av.                           |
| 11           | Low Titer        | 160        | 160             | 0.383                               |
| 12           |                  | 80         | 80              | 0.283                               |
| 13           |                  | 40         | 20              | 0.284                               |
| 14           |                  | 40         | 40              | 0.287                               |
| GMT*         |                  | 67         | 57              | 0.309 Av.                           |
| 15           | Negative         | 20         | 10              | 0.393                               |
| 16           |                  | 10         | 10              | 0.177                               |
| GMT*         |                  | 14         | 10              | 0.285 Av.                           |

GMT = Geometric mean titer

FIG. 2. Distribution of ELISA readings in normal sera (fresh unfrozen) and previously frozen sera from patients with high antibody titers to viruses other than EBV, and from patients with infectious mononucleosis with different levels of heterophile antibody as measured by the IAHA test.
Reproducibility

Aliquots of sera retested on the same day gave very closely comparable readings. Those stored at 4°C dropped in titer (as mentioned above). Another technician, working with the directions given, was able to reproduce the results after some initial difficulty in forming a good Rbc monolayer.

Quantitative Correlations

Twenty-two previously frozen sera which represented the peak heterophile titer by standard tests for each patient were tested by the ELISA test. The results are compared in Table 3 and Fig. 3. All sera had ELISA values over 0.25. The highest ELISA values generally correlated with the highest heterophile titers, but there was not an exact correlation, just as variations existed in titers among the other tests. This is not surprising, as each test used different red cells with somewhat different affinities for heterophile antibody.

DISCUSSION

An ELISA test for heterophile antibody has been described. The advantages of this assay are (a) it is simple to perform, (b) it has a wide margin of tolerance under various testing conditions, (c) it is easy to standardize, (d) it shows little susceptibility

| Serum Number | IAHA | Horse Rbc | Sheep Rbc | ELISA Reading | Average ELISA |
|--------------|------|-----------|-----------|----------------|---------------|
| 1            | 10240| 10540     | 2560      | 1.008          | N/A           |
| 2            | 10240| 10540     | 640       | 1.300          | N/A           |
| 3            | 10240| 5120      | 1280      | 1.129          | N/A           |
| 4            | 10240| 5120      | 320       | 0.763          | N/A           |
| 5            | 10240| 5120      | 640       | 0.584          | N/A           |
| 6            | 10240| 2560      | 320       | 0.794          | N/A           |
| 7            | 10240| 640       | 160       | 0.989          | N/A           |
| 8            | 10240| 160       | 40        | 0.880          | 0.931         |
| 9            | 5120 | 1280      | 160       | 0.787          | N/A           |
| 10           | 5120 | 640       | 320       | 0.820          | N/A           |
| 11           | 5120 | 640       | 160       | 0.434          | 0.680         |
| 12           | 2560 | 1280      | 320       | 0.747          | N/A           |
| 13           | 2560 | 640       | 80        | 0.556          | N/A           |
| 14           | 2560 | 320       | 40        | 0.410          | 0.680         |
| 15           | 1280 | 1280      | 80        | 0.689          | N/A           |
| 16           | 1280 | 160       | 80        | 0.524          | N/A           |
| 17           | 1280 | 160       | 40        | 0.351          | 0.521         |
| 18           | 640  | 320       | 80        | 0.631          | N/A           |
| 19           | 640  | 160       | 80        | 0.371          | 0.501         |
| 20           | 320  | 320       | 40        | 0.266          | 0.266         |
| 21           | 160  | 80        | 20        | 0.302          | N/A           |
| 22           | 160  | 80        | 40        | 0.425          | 0.363         |
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FIG. 3. Distribution of sheep Rbc, horse Rbc, and IAHA titers among 22 sera from infectious mononucleosis cases (A) as compared with ELISA readings (B); sera peak heterophile responses selected from 22 sets of serial samples from infectious mononucleosis cases.

ity to prozone effect, (e) no absorption of sera with guinea pig kidney is needed, (f) it has a sensitivity approaching or exceeding the IAHA [5] currently the most sensitive test developed [4], (g) a number of sera can be tested at the same time, and (h) a single dilution gives a good estimation of titer.

The main problems are two. The first is that it requires a microtiter reader. Since many laboratories are doing ELISA tests for other reasons, many may have a reader. If not, a color standard could be used and the plate can be read for qualitative semi-quantitative evaluation on white background with overhead illumination. A resolution equivalent to 0.150 to 0.200 can be achieved by this method. Second, the fresh bovine erythrocyte and Poly-L-Lysine solutions are relatively unstable and should be prepared every few weeks. Plates coated with poly-L-Lysine and a monolayer of beef Rbc may be held for several weeks at 4°C.

We compared ELISA readings with the results of the IAHA [5] and the absorbed horse hemagglutination test [3,4] readings on fresh IM and normal sera. For the ELISA test we used the Titretek Multi-Skan eight-channel microtiter spectrophotometer (Flow Labs, McLean, VA) with a 492 nm filter. On this basis we considered a reading of <0.25 as negative, from >0.25 to 0.35 as low positive, and values >0.35 readings as positive. The values must be determined in each laboratory.

Sera which had been frozen for variable periods and/or held at 4°C for a week gave ELISA values about half of that of fresh sera. Despite that, an ELISA reading of 0.25 or higher on either fresh or frozen sera was a reliable indicator of an elevated heterophile antibody titer. In practice, however, it may be desirable to use values of ≥3 standard deviations over comparable control sera (fresh or frozen) as the standard of positivity.

The presence of elevated antibody titer to other viruses (CMV, HSV, rubella, and
measles) resulted in normal or almost normal ELISA values in 16 of 17 sera—a specificity of 94 percent. The sensitivity of the ELISA test was high. All sera giving positive titers of ≥1:40 by the IAHA test, the most sensitive procedure we have tested, also had ELISA values of 0.25 or higher. One serum, negative by IAHA, also had a positive ELISA value of 0.39.

The ELISA reading at a single serum dilution of 1:20 gave a good, but not exact, correlation of the titer as measured by other heterophile antibody tests. As used by us, the ELISA measures predominantly IgM antibody; no increase in ELISA readings was seen with the addition of anti-IgA or IgG conjugates. This is consistent with the findings of Fletcher and Lo [7] that the PB antibody contains little IgG component. However, others have recently found a contribution of the IgG class [8].

During the preparation of this manuscript Halbert et al. [8] published a similar study on the use of the ELISA test for heterophile antibody. Their technique differed from ours in (1) use of purified bovine erythrocytes stroma as the antigen, (2) a solid-phase covalent bonding with small plastic discs containing isotheircyanate groups, (3) immunospecific heavy-chain goat anti-human IgM serum conjugated to purified calf intestinal alkaline phosphatase, (4) reading of the test in a vial rather than on a microtiter plate, and (5) reporting the test results as percentage of absorbance of a positive control standardized to give a reading of approximately 1.0 in the assay. They tested 150 normal sera by the ELISA test which were all negative and 79 heterophile-positive sera (horse Rbc test) from patients with infectious mononucleosis, of which 78 were positive by the ELISA test. The ELISA test was also weakly positive in sera from 14 IM patients that were horse Rbc-negative. The presence of EBV-IgM antibody or ox-hemolysin antibody indicated that ten of these represented true positives.

The results of this paper and that of Halbert et al. [8] suggest that an ELISA test for heterophile antibody can be quantitatively performed using a single serum dilution. The test is both specific and sensitive. As more laboratories adopt ELISA procedures for a variety of antibody and antigen detection methods, the use of one or the other of the heterophile antibody procedures may be found useful and practical.

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