Use of Stable, Sensitized Cells in Indirect Micro Hemagglutination Test for Amebiasis

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Human "O" cells were fixed with pyruvic aldehyde, treated with tannic acid, and fixed with glutaraldehyde. The cells were sensitized with amoeba antigen and stored in a refrigerator. The sensitized cells were used periodically for the indirect hemagglutination test with a battery of sera from patients with intestinal amebiasis and confirmed and unconfirmed amebic liver abscess, and also from negative controls. The same battery was tested with cells sensitized with different batches of antigen and also with fresh sheep cells. None of the cells showed any reaction with negative control sera. The fixed cells remained sensitive and stable throughout the study. Reproducibility of the titers with the fixed cells within each day and from day to day was satisfactory. The titers with fixed human "O" cells were slightly lower than were the titers with fresh sheep cells. The advantages of using stable, sensitized cells are pointed out.

The indirect hemagglutination (IHA) test has been shown to be a sensitive and specific method for detection of antibodies to Entamoeba histolytica (7). The application of this method in clinical amebiasis was evaluated earlier in our laboratory (5, 10), and the test has also been used for seroepidemiology studies (4). There are, however, some limitations to the use of the test. Preparation and sensitization of the red blood cells is time consuming and day-to-day variation is not unusual. It has been shown that several factors, such as the type of plastic tray, antiserum diluent, microtiteration loops, antigenic variation, and the use of various batches of red blood cells, influence the IHA titer in some systems (6). The present study was undertaken to try to overcome some of these problems.

MATERIALS AND METHODS

Buffer. Phosphate-buffered saline (PBS) consisted of 0.06 M Na₂HPO₄ and NaH₂PO₄ which contained 0.74% NaCl. Sodium oxide (0.1%) was added as a preservative. The pH of this buffer was 6.9.

Preparation of erythrocytes. One- or two-day-old human "O" cells, collected in modified Alsever's solution (glucose, 20.5 g; sodium citrate, 8.0 g; sodium chloride, 4.2 g; and citric acid, 0.55 g in 1 liter of distilled water, pH adjusted to 6.1), were washed in PBS three times. To a 4% suspension of the cells in PBS, an equal volume of 2% pyruvic aldehyde (Ptaltz & Bauer, Flushing, N.Y.) was added slowly while the cells were being mixed on a magnetic stirrer for 30 min at room temperature. They were then left for 18 h at 4 C and occasionally stirred. Then, the cells were centrifuged at 700 × g for 10 min and washed three times in 10 volumes of PBS. An equal volume of 2.5% cell suspension in PBS was mixed with a 1:2,500 solution of tannin acid in PBS at 4 C for 30 min. The tanned cells were centrifuged and washed once in PBS. An equal volume of 4% washed, tanned cells was mixed with a 2% glutaraldehyde (Fisher Scientific Co.) solution in PBS on a magnetic stirrer for 2 h at room temperature; then the cells were centrifuged and washed three times in PBS. A 10% cell suspension was prepared and kept at 4 C until used.

Antigen. The antigen used in this study was made from the HK9 strain of E. histolytica (obtained from L. S. Diamond) and grown in Diamond axenic medium (2). Amoebae (4,000,000/ml) were washed several times with saline and then suspended in sterile, distilled water. The suspension was sonically treated with a Branson Sonic Power model S75 sonic oscillator (power set at no. 6) until microscopy examination verified complete disruption of the organisms. The sonicate extract was centrifuged at 17,000 × g for 30 min at 4 C. The supernatant then was lyophilized in small samples (0.5 ml). Sensitization and preparation of the fixed cells for the IHA test has been described before (3).

Sensitization of the cells. One part of fixed, tanned, and packed cells was suspended in 10 parts of an appropriate dilution of antigen (vol/vol). The suspension was incubated in a 50 C water bath for 5 min and then at 4 C for 16 h. Then the cells were suspended and incubated in a 50 C water bath an additional 5 min. A 1% suspension of sensitized cells was prepared by adding PBS containing 0.25% bovine serum albumin, fraction V (Armour Pharmaceutical Co.); it was kept at 4 C until used.

Diluent. The diluent for the micro IHA test was prepared by mixing 2.5 ml of 0.2 M Na₂HPO₄·7H₂O...
and 2.5 ml of 0.2 M NaH₂PO₄, H₂O with 7.5 ml of distilled water. This diluent usually contained 0.75% of normal rabbit serum. However, the concentration of normal rabbit serum could be varied from 0.25 to 1.00% in order to obtain desirable IHA patterns and titers for each batch of fixed, sensitized cells.

**Antisera.** Twenty serum samples were used in this study. Five (no. 1 to 5) were obtained from patients with parasitologically proven intestinal amebiasis; one (no. 6) was obtained from a patient with both a liver abscess and intestinal amebiasis, and three (no. 7 to 9) from patients with confirmed liver abscess (i.e., organism identified in aspirate fluid). Serum no. 7 had been known to be nonreactive in the IHA test. Five (no. 10 to 14) of the samples were from patients in whom amebic liver abscess was not confirmed. Serum no. 15 was submitted for routine serology with no clinical information provided. Serum no. 16 was from an asymptomatic carrier whose stool was positive. Serum no. 17 was from a clinically suspected patient whose stool was negative. Three negative control sera (no. 18 to 20) also were included in the study. The sources of sera and clinical information are shown in Table 1.

In order to test the reproducibility of the test within a given day and from day to day, we divided some of the sera into samples, so that several replicates of the same serum were tested in each run. All of the samples in a run were coded prior to the run. Forty-six samples were tested in each run. Of the 20 sera listed in Table 1, one serum was repeated five times, eight sera three times, and six sera twice. The remaining five sera were tested only once in each run.

In order to determine the effect of a different batch of cells (from the same donor) and a different batch of antigen on the titer, we used two additional batches of cells (lots no. 1 and 2).

**IHA tests.** Disposable (autotray) plastic plates with 120 U-shaped wells, (obtained from Canalclo, Rockville, Md.) were used for all tests, except for those with fresh sheep cells; then disposable plastic plates with 96 U-shaped wells (Linbro Chemical Co., New Haven, Conn.) were used. Into each well, we dropped 0.05 ml of diluent with a dropper obtained from Cooke Engineering Co., Alexandria, Va. We transferred 0.05 ml of serum into each of the first wells by using a Takatsky “tulip-type” loop (Cooke Engineering Co.). Twofold dilutions of sera were made, starting with a 1:2 dilution. The loops were presoaked in diluent before the sera were transferred to prevent the carry-over effect. The plates were placed on a shaker while 0.025 ml of a stable, sensitized cell suspension was dropped into each well. The IHA patterns were read after 1 to 2 h of incubation at room temperature.

**RESULTS**

Fourteen sera gave positive titers with the fresh cells. The titers of these sera both with fresh cells and with stable, sensitized cells are shown in Table 2. Because the three negative control sera were negative each time tested, there was no false positives with the stable, sensitized cells or with the fresh cells. The serum from the asymptomatic person (no. 16) was negative; the serum (no. 15) submitted with no clinical information was positive.

The stable cells in the first run showed a very

| Serum No. | Code  | Source             | Culture of stool | Clinical information                     |
|-----------|-------|--------------------|------------------|------------------------------------------|
| 1         | K3205 | K. Juniper, Ark.   | +                | Colon amebic ulcer                       |
| 2         | K3170 | K. Juniper, Ark.   | +                | Ameboma of colon                         |
| 3         | 6J    | K. Juniper, Ark.   | +                | Amebic colitis                           |
| 4         | K3221 | K. Juniper, Ark.   | +                | Chronic amebiasis                        |
| 5         | 25    | South Africa       | +                | Symptomatic, amebic ulcer                |
| 6         | 32    | Robinson, England  | +                | Colon and liver abscesses                |
| 7         | 9C    | K. Juniper, Ark.   |                  | Confirmed liver abscesses                |
| 8         | 12B   | Robinson, England  |                  | Confirmed liver abscesses                |
| 9         | 29    | Elsdon-Dew, South Africa |              | Confirmed liver abscesses                |
| 10        | K8831 | Naval Hospital, Oakland, Calif. |       | Confirmed liver abscesses                |
| 11        | K7007 | Air Force Hospital, Fla. |              | Confirmed liver abscesses                |
| 12        | K4376 | Scott Air Force Base, Ill. |           | Confirmed liver abscesses                |
| 13        | K069  | Florida            |                  | Unconfirmed liver abscesses              |
| 14        | 24    | Robinson, England  |                  | Unconfirmed liver abscesses              |
| 15        | K5759 | Phoenixville, Pa.  |                  | Unconfirmed liver abscesses              |
| 16        | K1937 | K. Juniper         | +                | Asymptomatic                             |
| 17        | 26    | Biagi, Mexico      |                  | Suspected intestinal amebiasis           |
| 18        | A.T.  | Atlanta, Ga.       |                  | Asymptomatic, negative control           |
| 19        | 31    | Atlanta, Ga.       |                  | Asymptomatic, negative control           |
| 20        | 33    | Atlanta, Ga.       |                  | Asymptomatic, negative control           |

* Known negative control.
### Table 2. Indirect hemagglutination titers using stable and fresh cells

| Serum no. | Replicate | O(A) | 4(B) | 11(C) | 30(D) | 45(E) | O(F) | O(G) | 0 |
|-----------|-----------|------|------|-------|-------|-------|------|------|---|
| 1         | I         | 1,024 | 2,048 | 512   | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 |
| 1         | II        | 1,024 | 2,048 | 1,024 | 512   | 1,024 | 1,024 | 1,024 | 2,048 |
| 1         | III       | 4,096 | 512   | 4,096 | 256   | 1,024 | 256   | 256   | 2,048 |
| 1         | IV        | 4,096 | 1,024 | 2,048 | 1,024 | 512   | 1,024 | 512   | 1,024 |
| 1         | V         | 2,048 | 1,024 | 512   | 1,024 | 512   | 1,024 | 2,048 | 1,024 |
| 2         | I         | 32,768| 2,048 | 2,048 | 2,048 | 4,096 | 1,024 | 1,024 | 2,048 |
| 2         | II        | 4,096 | 2,048 | 2,048 | 2,048 | 4,096 | 1,024 | 1,024 | 2,048 |
| 2         | III       | 32,768| 2,048 | 2,048 | 2,048 | 4,096 | 1,024 | 1,024 | 2,048 |
| 3         | I         | 32,768| 2,048 | 2,048 | 2,048 | 4,096 | 1,024 | 1,024 | 2,048 |
| 3         | II        | 4,096 | 2,048 | 4,096 | 2,048 | 512   | 1,024 | 2,048 | 1,024 |
| 3         | III       | 2,048 | 1,024 | 1,024 | 2,048 | 2,048 | 1,024 | 1,024 | 2,048 |
| 4         | I         | 32,768| 2,048 | 2,048 | 1,024 | 512   | 1,024 | 1,024 | 2,048 |
| 4         | II        | 32,768| 1,024 | 2,048 | 512   | 2,048 | 512   | 2,048 | 4,096 |
| 5         | I         | 512   | 128   | 256   | 128   | 128   | 512   | 256   | 512 |
| 5         | II        | 128   | 256   | 256   | 128   | 512   | 256   | 256   | 512 |
| 6         | I         | 2,048 | 256   | 256   | 512   | 512   | 2,048 | 512   | 256 |
| 6         | II        | 2,048 | 256   | 2,048 | 512   | 1,024 | 1,024 | 1,024 | 2,048 |
| 8         | I         | 2,048 | 1,024 | 512   | 2,048 | 2,048 | 1,024 | 1,024 | 2,048 |
| 8         | II        | 2,048 | 1,024 | 512   | 2,048 | 2,048 | 1,024 | 1,024 | 2,048 |
| 9         | I         | 32,768| 4,096 | 1,024 | 2,048 | 2,048 | 1,024 | 2,048 | 1,024 |
| 9         | II        | 32,768| 4,096 | 2,048 | 2,048 | 1,024 | 1,024 | 1,024 | 2,048 |
| 10        | I         | 2,048 | 1,024 | 512   | 2,048 | 2,048 | 1,024 | 1,024 | 2,048 |
| 10        | II        | 1,024 | 512   | 2,048 | 512   | 1,024 | 1,024 | 512   | 1,024 |
| 10        | III       | 1,024 | 512   | 1,024 | 512   | 1,024 | 1,024 | 512   | 1,024 |
| 11        | I         | 2,048 | 2,048 | 512   | 1,024 | 512   | 1,024 | 512   | 4,096 |
| 11        | II        | 512   | 512   | 1,024 | 256   | 512   | 512   | 512   | 2,048 |
| 12        | I         | 64    | 32    | 32    | 64    | 32    | 128   | 128   | 128 |
| 12        | II        | 128   | 64    | 32    | 64    | 32    | 128   | 64    | 128 |
| 13        | I         | 512   | 512   | 256   | 64    | 64    | 128   | 64    | 512 |
| 13        | II        | 256   | 32    | 128   | 128   | 256   | 256   | 256   | 512 |
| 14        | I         | 1,024 | 256   | 1,024 | 512   | 1,024 | 1,024 | 1,024 | 2,048 |
| 14        | II        | 1,024 | 256   | 1,024 | 512   | 1,024 | 1,024 | 1,024 | 2,048 |
| 15        | I         | 1,024 | 512   | 512   | 512   | 512   | 256   | 256   | 512 |
| 15        | II        | 1,024 | 512   | 512   | 512   | 512   | 256   | 256   | 512 |
| 15        | III       | 512   | 256   | 512   | 512   | 256   | 512   | 512   | 512 |

*a Days after preparation (and run).*

High titer (32,000) with four sera on five occasions. These high titers did not occur in other runs for the same samples. Titers varied for some sera more than for others. For example, with serum no. 15, which was repeated three times in each run, an identical titer (512) was obtained from 16 of the 24 trials, and there was a two dilution difference between the highest and the lowest titer. Serum no. 16, which was tested only once in each run, showed a four-dilution difference between the highest and the lowest titer.

In all cases but one, titers obtained with fresh cells were higher than titers obtained with stable, sensitized cells that had been stored for 30 days. On the average, titers with stable cells (after 30 days of storage) were 1.3 dilutions lower than the titers with the fresh cells. This average was 1.2 dilutions lower when the cells were stored for 45 days.

**Intestinal amebiasis.** All of the sera from persons with confirmed intestinal amebiasis showed antibodies with both the fresh and stable cells, but the serum sample from the patient suspected of having intestinal amebiasis (no. 17) was negative with both stable and fresh cells.

**Liver abscess.** Of the three serum samples from patients with confirmed liver abscess, two were positive (no. 8 and 9), but the other (no. 7) was negative with both types of cells. Serum no. 7 has been tested in other laboratories that perform the IHA test for amebiasis and has been consistently negative. Three of the five
serum samples from patients in whom liver abscess was unconfirmed were positive both with stable cells and fresh cells. Serum no. 13 was positive with the fresh cells, but had titers of lower than 128 three out of ten times with the stable cells. Serum no. 12 was a borderline positive with the fresh cells, but gave titers of lower than 128 nine out of ten times with stable cells.

**DISCUSSION**

Statistical analysis of the data in Table 2 showed that the within-run reproducibility of the titers of sera from persons with intestinal amebiasis (1, 2, 3, 4, and 5) was not significantly different from that of the titers of sera from persons with liver abscesses (8, 10–13). Within-run reproducibility of run D did not differ from that obtained in run F, or G, or for titers obtained with the fresh sheep cells. There were also no significant differences in within-run reproducibility of the titers in runs A, B, C, D, and E. However, within-run reproducibility of the titers obtained in run A differed significantly from within-run reproducibility of the titers obtained with the fresh cells. Statistical analysis of the data for sera no. 1 to 5, 10 to 13, and 15 (positive sera replicated more than once in each run) also showed that there were no significant differences in the titers among runs A, B, C, D, E, F, or G. The only significant difference was found between fresh cells and run A of the stable cells. This indicates that run A was different from the run with fresh cells. The difference seems to be mainly due to the five very high titers obtained for four sera in run A. Four of the sera with high titers were located on the same plastic plate in run A. This indicates that an aberrant plate may have been the cause of the high titer.

The use of glutaraldehyde-fixed sheep erythrocytes in the IHA test for amebiasis has been reported by Krupp (8). Her studies showed that the titers for four sera remained constant for a period of 1 month if the glutaraldehyde-fixed and sensitized cells were shell frozen and stored at −70 C. Her attempts to lyophilize the cells were not successful.

We believe the present method of preparation of stable, sensitized cells is superior to one-step glutaraldehyde fixation used by Krupp. The cells prepared by our method remain stable for longer periods of time than the cells prepared by glutaraldehyde fixation alone. One batch of cells in our laboratory retained sensitivity for more than 1 year after their preparation. The cells prepared by our method show no clumping, and their pattern is superior to the pattern of the cells prepared by one-step glutaraldehyde fixation. In addition, the cells prepared by the present procedure do not have to be stored at −70 C. Even storage at room temperature for a few hours does not seem to affect their sensitivity, and thus facilitates their use and shipment.

Comparison of the titers between fresh sheep cells and stable human "O" cells shows that, in general, stable cells yield titers that are one or two dilutions lower than those of the fresh cells. This has also been reported by Krupp (8). At present in our laboratory, titers of 128 or greater with the fresh sheep cells are considered positive. Additional experiments with stable cells will determine what titers should be considered positive when these cells are used for the IHA test for amebiasis.

In the IHA test for amebiasis, stable, sensitized cells should provide a more reliable system than the various batches of fresh red blood cells. Variation in surface characteristics of different batches of red blood cells has been reported (11). Fragility and susceptibility to lysis differ from cell batch to cell batch (1), and even the same batch of cells undergoes physiological and metabolic changes during storage (9).

In laboratories where the IHA test is routinely performed, considerable time can be saved in the daily preparation, sensitization, and pretesting of cells if stable cells are used. In addition, the cells can be prepared in a central laboratory and shipped to other places for use (where there is no facility for preparation of amoeba antigen).

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