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# USE OF STABLE SENSITIZED CELLS IN INDIRECT MICROHEMAGGLUTINATION TEST FOR MALARIA

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# USE OF STABLE SENSITIZED CELLS IN INDIRECT MICROHEMAGGLUTINATION TEST FOR MALARIA*

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*Abstract.* Human "O" erythrocytes were stabilized with pyruvic aldehyde, tanned, and fixed with glutaraldehyde. These cells were sensitized with antigenic preparations of *Plasmodium knowlesi* and were used in the indirect microhemagglutination test with serum samples from human malaria cases and negative controls. The cells did not lose sensitivity after 3 months of storage. The practical application of these cells in serocpidemiological studies of malaria is pointed out.

Lack of reproducibility is one of the inherent limitations of the indirect microhemagglutination (IHA) test. Hirata et al. have reported that the type of plastic tray, antiserum diluent, and microtitration loops used influence the reproducibility of the IHA titers.[1]

Both uniformity of size and availability make red blood cells very useful for IHA tests. However, the variation in lots of red blood cells, differences in surface characteristics of various batches of erythrocytes,[2] fragility, susceptibility to lysis,[3] and physiologic and metabolic changes during storage[4] are some of the difficulties associated with their use.

Another important factor influencing the malaria IHA test is the use of complex antigenic materials for cell sensitization. If the combined effect on titer fluctuation due to differences in red blood cells and antigenic variability is eliminated, the reproducibility of the technique will be greatly increased. In order to accomplish this end for the malaria IHA test, the present study was undertaken.

## MATERIALS AND METHODS

*Buffer.* Phosphate buffered saline (PBS) consisted of 0.06 M $Na_2HPO_4$ and $NaH_2PO_4$ which contained 0.74% NaCl. Sodium azide (0.1%) was added as a preservative. The pH of this buffer was 6.9.

*Preparation of erythrocytes.* One- or 2-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; citric acid 0.55 g in one 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 (Pfaltz & 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 18 hrs at 4°C and occasionally stirred. Then, the cells were centrifuged at 700 × g for 10 min and washed three times in 10 volume of PBS. An equal volume of 2.5% cell suspension in PBS was mixed with a 1:2,500 solution of tannic 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 the magnetic stirrer for 2 hours 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 prepared from *Plasmodium knowlesi* by slight modification of the method described by Rogers et al.[5] The frozen parasites were thawed and diluted 1:4 with PBS and sonified continuously in a Biosonik IIA (Brownwill Scientific, Rochester, N.Y.) for 7 min at room temperature with the microprobe set at ½ power. Microscopic

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examination of this suspension after staining showed complete disruption of parasite cells. The suspension was centrifuged at 12,100 × g for 15 min. The supernatant fluid was the antigen, and it was stored at −70 C until used. This antigen was titrated, and the dilution that gave the highest titer with a battery of positive sera and no nonspecific reactions with negative sera was used to sensitize cells. On an average the antigen could be diluted tenfold. All dilutions were made with PBS.

*Sensitization of the cells.* One part of fixed, tanned, and packed cells was suspended in 10 parts of an appropriate dilution of antigen (V/V). The suspension was incubated in a 50 C water bath for 5 min and then at 4 C for 16 hr. Then the cells were suspended and incubated in a 50 C water bath for 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 $Na_2HPO_4 \cdot 7H_2O$ and 2.5 ml of 0.2 M $NaH_2PO_4 \cdot H_2O$ with 7.5 ml distilled water. This diluent usually contained 0.75% of normal rabbit serum (NRS). However, the concentration of NRS could be varied from 0.25 to 1.0% in order to obtain desirable IHA patterns and titers for each batch of fixed sensitized cells.

*Serum samples titrated.* Two batteries of sera were tested. Battery A consisted of 24 serum samples. Twenty-one of the sera were collected in October 1970 from people coming to a malaria fever clinic in Cuiaba, Mato Grosso, Brazil. All but one of these individuals were adults. Seventeen of the 21 showed parasites in their blood smears; 10 of the parasites were identified as *Plasmodium falciparum*, 6 as *P. vivax*, and 1 was unidentified. Three serum samples were received from the New York State Public Health Laboratory and were used as negative controls. Battery B consisted of 40 samples. All of the positive samples in this battery were from slide-proven cases of malaria. The sera were submitted to the Center for Disease Control in Atlanta, Ga., by various military organizations and State Health Laboratories.

*Indirect IHA test.* Disposable plastic plates with 96 U-shaped wells were obtained from Linbro Chemical Co., New Haven, Conn. 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 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 hours of incubation at room temperature, according to the method of Stavitsky.[7]

TABLE 1

*IHA titers of Battery A serum samples with stable sensitized cells*

| No. | Code | Days after preparation of cells | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 5 | 13 | 20 | 90 |
| 1 | 1589 | 128 | 64 | 64 | 128 | 128 | 32 |
| 2 | 1669 | 1,024 | 2,048 | 1,024 | 1,024 | 1,024 | 512 |
| 3 | 1638 | 2,048 | 1,024 | 2,048 | 1,024 | 1,024 | 1,024 |
| 4 | 1676 | 512 | 256 | 256 | 256 | 512 | 256 |
| 5 | 1654 | 4,096 | 4,096 | 4,096 | 4,096 | 4,096 | 4,096 |
| 6 | 1653 | 4,096 | 2,048 | 2,048 | 256 | 2,048 | 1,024 |
| 7 | 1657 | 1,024 | 512 | 512 | 512 | 512 | 256 |
| 8 | 1659 | 512 | 512 | 512 | 512 | 512 | 512 |
| 9 | 1666 | 2,048 | 512 | 512 | 512 | 512 | 512 |
| 10 | 1642 | 512 | 512 | 1,024 | 512 | 1,024 | 512 |
| 11 | 1597 | 1,024 | 512 | 512 | - - | 256 | 256 |
| 12 | 1686 | 1,024 | 1,024 | 512 | 512 | 512 | 256 |
| 13 | NY1033 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 1674 | 64 | 64 | 64 | 128 | 64 | 64 |
| 15 | 1717 | 4,096 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 |
| 16 | C-74 | 2,048 | 1,024 | 2,048 | 1,024 | 2,048 | 1,024 |
| 17 | 1605 | 256 | 256 | 256 | 512 | 256 | 256 |
| 18 | NY1005 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19 | 1601 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 |
| 20 | 1688 | 128 | 128 | 64 | 128 | 128 | 64 |
| 21 | NY1023 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | 1634 | 4,096 | 2,048 | 2,048 | 1,024 | 1,024 | 512 |
| 23 | 1725 | 4,096 | 1,024 | 1,024 | 1,024 | - - | 1,024 |
| 24 | 1604 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 | 1,024 |

## RESULTS

For each serum in battery A, the geometric mean and the distribution of titers about that geometric mean were determined. Ninety-two percent of the 118 replicate titers on 20 sera (sera no. 5, 13, 18, and 21 from Table 1 were omitted in these calculations) were within one dilution factor (twofold) of their respective within serum geometric means.

TABLE 2

*HIA titers of Battery B sera with stable sensitized cells*

| No. | Code | Days after preparation of cells | | | |
|---|---|---|---|---|---|
| | | 0 | 30 | 60 | 90 |
| 1 | 69 | 32 | 32 | 32 | 32 |
| 2 | 66 | 4,096 | 4,096 | 4,096 | 4,096 |
| 3 | 73 | 0 | 4 | 0 | 0 |
| 4 | 74 | 0 | 0 | 0 | 0 |
| 5 | 75 | 256 | 128 | 256 | 128 |
| 6 | 76 | 128 | 128 | 256 | 128 |
| 7 | 77 | 256 | 512 | 256 | 256 |
| 8 | 91 | 128 | 64 | 128 | 64 |
| 9 | 93 | 4,096 | 4,096 | 4,096 | 4,096 |
| 10 | 94 | 0 | 0 | 0 | 0 |
| 11 | 34 | 256 | 128 | 128 | 128 |
| 12 | 33 | 32 | 16 | 16 | 16 |
| 13 | 10 | 4,096 | 4,096 | 4,096 | 4,096 |
| 14 | 29 | 128 | 64 | 128 | 128 |
| 15 | 57 | 512 | 256 | 256 | 256 |
| 16 | 35 | 0 | 0 | 0 | 0 |
| 17 | 52 | 2,048 | 4,096 | 4,096 | 4,096 |
| 18 | 53 | 128 | 256 | 128 | 128 |
| 19 | 37 | 0 | 0 | 0 | 0 |
| 20 | 32 | 256 | 256 | 256 | 256 |
| 21 | 166 | 1,024 | 1,024 | 1,024 | 1,024 |
| 22 | 174 | 512 | 256 | 256 | 128 |
| 23 | 146 | 512 | 512 | 256 | 128 |
| 24 | 152 | 1,024 | 2,048 | 1,024 | 2,048 |
| 25 | 149 | 512 | 512 | 512 | 512 |
| 26 | 162 | 512 | 512 | 256 | 256 |
| 27 | 173 | 0 | 0 | 0 | 0 |
| 28 | 181 | 1,024 | 1,024 | 1,024 | 1,024 |
| 29 | 178 | 8 | 8 | 0 | 8 |
| 30 | 176 | 4,096 | 2,048 | 4,096 | 4,096 |
| 31 | 116 | 512 | 256 | 512 | 256 |
| 32 | 145 | 4,096 | 4,096 | 4,096 | 4,096 |
| 33 | 114 | 4,096 | 4,096 | 4,096 | 4,096 |
| 34 | 113 | 0 | 0 | 0 | 0 |
| 35 | 99 | 256 | 512 | 512 | 512 |
| 36 | 98 | 4,096 | 4,096 | 4,096 | 4,096 |
| 37 | 135 | 4,096 | 4,096 | 4,096 | 4,096 |
| 38 | 137 | 1,024 | 1,024 | 1,024 | 1,024 |
| 39 | 97 | 256 | 512 | 256 | 256 |
| 40 | 139 | 4,096 | 4,096 | 4,096 | 4,096 |

Six replicate titers were obtained for each of 19 sera in Table 1 (sera no. 5, 6, 15, 22, and 23 were omitted from these calculations). For 6 of the 19 sera, these replicate titers were identical; for 7 sera, the highest titer was two times the lowest titer; and for the remaining 6 sera, the highest titer was four times the lowest titer.

Ninety-seven percent of 96 replicate titers for 24 sera in Table 2 (sera no. 2, 4, 9, 10, 13, 16, 17, 19, 27, 30, 32, 33, 34, 36, 37, and 40 were not included in these calculations) were within one dilution factor of the respective within serum geometric means.

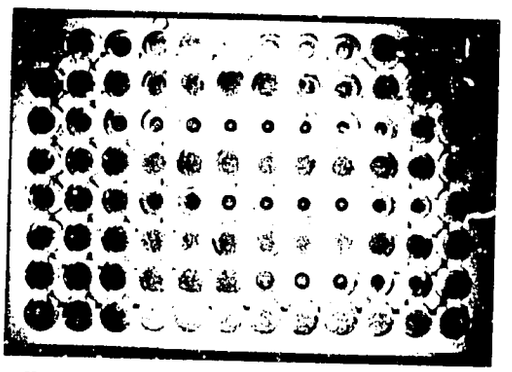

FIGURE 1. Hemagglutination patterns of malaria sensitized stable cells in serially diluted malaria antisera. The antisera were diluted from left to right. A negative reaction is shown by small circle, and a positive reaction is shown by a smooth or irregular mat shape.

Four replicate titers were obtained for each of 30 sera in Table 2 (sera no. 2, 9, 13, 17, 30, 32, 33, 36, 37, and 40 were not included in the calculations). For 12 of 30 sera, these replicate titers were identical; for 14 sera the highest titer was two times the lowest titer; for 2 sera the highest titer was four times the lowest titer; and for the remaining 2 sera the highest titer was more than four times the lowest titer.

The hemagglutination patterns of the stable sensitized cells were easy to read because the cells retained their original shape after stabilization, tanning, and sensitization (Fig. 1).

## DISCUSSION

The specificity and cross-reactivity of the antigen used in this study and in the HIA test with fresh cells have been evaluated with the sera from other parasitic diseases, syphilitic sera, and normal healthy human donors in an earlier study in this laboratory.[5] It was found that titers of 16 or greater occurred in 0.9% of the cases.

Some batches of human type "O" cells gave better results than others with a given batch of antigen. This occurs with fresh cells and has also been reported by Hirata and Brandriss for aldehyde fixed cells.[7]

Ling has compared stabilization of human "O" cells with several aldehydes and subsequent sensitization with protein antigens.[8] He concluded

that pyruvic aldehyde and glutaraldehyde were superior reagents for this purpose.

We have used each of these two aldehydes alone with only partial success. The combination of the two aldehydes, separated by the tanning procedure, proved to be a satisfactory method for stabilization of malaria antigen. Our attempts to couple malaria antigen to red blood cells before or after fixation by various chemical agents such as bis-diazotized benzidine,[9] chromic chloride,[10] carbodiimide,[11] difluorodinitrobenzene,[12] or cyanuric chloride[13] were not satisfactory. With some of these reagents only partial success was achieved and low titers resulted.

The difficulty encountered in preparing stable sensitized cells may be due to the complex nature of malaria antigens. Chavin reported that a saline extract of *Plasmodium berghei* contains, in addition to some lipids, eight to 10 proteins determined by precipitin assay and up to 15 proteins by polyacrylamide gel electrophoresis.[14] *P. knowlesi* cell-free extract showed 8 precipitin bands by agar gel and 13 bands by polyacrylamide gel electrophoresis.[15]

It has been shown that aldehydes will react with free amino groups of proteins.[16] In addition to amino groups, sulfhydryl groups and to some extent imidazole groups will react with aldehydes. The surface of the red blood cells becomes more anionic (when the free amino groups are bound by aldehyde) so they will react with the ionized groups of protein antigen. It is possible that aldehydes in addition to tannic acid treatment are responsible for retaining the complex malarial antigen on the cells surface in our experiments.

The aldehyde-fixed erythrocytes will provide a population of stable sensitized cells which can be used repeatedly until exhausted. This will save considerable time in the daily operation of laboratories that perform IHA tests routinely. Furthermore, the stable sensitized cells can be sent to other laboratories around the world without damage to the cells or loss of antigenicity.

The use of human type "O" cells in the IHA test obviates the need to absorb sera for heterophile antibodies. Variation found in different aliquots of red blood cells can be prevented by the use of one batch of aldehyde-fixed cells.

In this procedure a crude malaria antigen preparation was used. The time-consuming and tedious fractionation by column chromatography is not necessary to prepare a reactive antigen, and the loss of antigenicity due to fractionation procedures is eliminated.

Since small amounts of sera and antigen are required to perform the micro IHA test and large numbers of sera can be tested in a short period of time, the technique can be used as a practical tool in the seroepidemiology of malaria around the world.

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