Discussion

Susceptibility to meningococcal meningitis has been shown to be related to the absence of pre-existing serum antibodies (Goldschneider, Gotschlich & Artenstein, 1969). Although susceptible army recruits in the USA showed no evidence of immunoglobulin deficiency, there is some evidence from studies of young children and of persons with hypogammaglobulinaemia that immunodeficiency, especially of IgM, may be a predisposing factor. The results obtained in this study show no immunoglobulin deficiency associated with epidemic meningococcal meningitis in Africans. The findings therefore resemble those obtained in the American study, and extend them to a different population and to another immunoglobulin—namely, IgE—which may play some role in protective immunity in the respiratory tract. Recent studies have indicated that factors such as climate, dryness of the air, and seasonal overcrowding may favour the spread of infection in Africans (Ghipponi et al., 1971). Since there is no evidence of immunodeficiency associated with meningitis in Africans, at least in terms of a lack of serum immunoglobulins, there is no reason to suppose that control of the disease by an effective vaccine may not be possible.

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A Capillary Tube Agglutination Test for Malaria

by H. F. Kortmann, J. Lelijveld, J. P. J. Ross & K. F. Lohr

A number of serological techniques have been used for assessing circulating antibodies in subjects with current or previous malarial infections, and their application to the study of malaria has been reviewed by Voller & Bruce-Chwatt (1968). Most of the older tests such as precipitation, agglutination, and complement fixation have not proved completely satisfactory, and of the newer techniques the indirect haemagglutination test has yet to be thoroughly established; immunofluorescence tests alone have been developed as a practicable diagnostic aid. Many of these sero-diagnostic methods are, however, of limited value because they are too complicated or require elaborate laboratory equipment and skilled technical supervision.

The capillary-tube agglutination (CA) test avoids many of these difficulties and has been successfully employed to detect antibodies of several animal protozoan parasites. Ristic (1962) first described a CA test for the diagnosis of bovine anaplasmosis, using as antigen particulate suspensions of infected blood disrupted by ultrasonic oscillation. The stabi-
lity of these antigens, which are easily transported and can be stored for several months at 4°C, greatly extends the scope of the test. Although capillary tubes were used by Kreier, Pearson & Stilwill (1965) in an agglutination test for *Plasmodium gallinaceum*, their antigen consisted of whole parasites and suffered from many of the disadvantages of the older tests. However, Ristic’s basic method of preparing antigens was subsequently modified to detect antibodies to *Babesia bigemina* (Löhr & Ross, 1969), the *Trypanosoma brucei* subgroup and *T. rhodesiense* (Ross, 1971), and *Theileria parva* and *T. mutans* (Ross & Löhr, unpublished data).

The present report assesses the feasibility of employing ultrasonic techniques to establish a CA test for *Plasmodium falciparum*.

**Materials and methods**

**Preparation of antigen.** Red blood cells infected with schizonts and trophozoites of *P. falciparum* were harvested in blood from heavily infected human placentae at the maternity clinic of the Magila Mission Hospital. The hospital is situated in an area of North-Eastern Tanzania that is highly endemic for malaria. Extraction was commenced immediately after delivery of the placenta by making cuts in the maternal surface about 20 mm deep and gently squeezing and washing the blood into phosphate-buffered saline (PBS) at pH 7.2. The suspension was made up to 250 ml and centrifuged at 1 000 g for 15 minutes. The supernatant was discarded. The residue was resuspended in 250 ml of PBS and the washing process was repeated three times. A brownish top layer of between 10 and 15 ml comprising 20–70% of the infected erythrocytes was removed from the final residue. Each collection was then pooled and stored at −70°C.

When required, 50-ml samples were thawed and disintegrated by ultrasonic oscillation for 5 minutes (20 kHz, 100 W, using a 9.5-mm probe at maximum amplitude). Operating temperatures were kept low by circulating chilled water through the treatment chamber. The resulting suspension was then centrifuged at 108 000 g for 30 minutes, a temperature of 4°C being maintained. The resulting pellet showed two distinct layers; the thicker bottom layer, which was dark-brown in colour and composed mostly of haemoglobin pigment, was discarded. The upper, reddish brown layer was retained and homogenized in a tissue grinder. The homogenate was then made up to 50 ml with PBS and recentrifuged at 108 000 g. This washing process was repeated until the supernatant was clear and there was no further evidence of a dark-brown lower layer. The final pellet was homogenized in 3–5 times its own volume of barbital-buffered saline at pH 7.2 and centrifuged at 1 000 g for 30 seconds. The supernatant, which was the antigen, was preserved by adding 40% formol to a concentration of 0.02%. The antigen was then dispensed into 1-ml vials and stored at 4°C until required.

The disintegration of infected material released into suspension large amounts of haemoglobin pigment granules and their removal was essential since they agglutinated spontaneously and obscured specific reactions. Separation was difficult however, and although it was possible to remove most of the pigment by differential centrifugation, there was currently a considerable loss of antigenic material and a disappointing harvest of effective antigen. For this reason a satisfactory antigen was produced only from highly infected material in which at least 40% of the erythrocytes were parasitized. Material with lower parasitaemias produced antigens of poor quality that failed to agglutinate many of the positive control sera. From a total of 40 malaria-infected placentae, five batches of antigen were prepared of which only two, derived from the most heavily parasitized material, were satisfactory. As observed with bovine CA antigen preparations, the exposure time to ultrasonic oscillation was highly critical. Insufficient treatment resulted in antigens that reacted nonspecifically, giving false positive reactions, while overexposure progressively destroyed the antigenicity (Löhr & Ross, 1969; Ross, 1971). Of the two satisfactory batches of antigen, one was later inadvertently destroyed in this manner. The remaining batch, consisting of 3 ml of antigen, was produced from the pooled extracts of 3 infected placentae and was sufficient for about 750 tests and adequate for the evaluation described below.

**Test procedure.** The test is carried out in capillary tubes 90 mm long, with a bore of 0.5 mm. Antigen is drawn into the tube by capillarity to a length of about 10 mm and this is followed by the test serum, full strength or diluted, until the tube is full. The sera are previously inactivated in a water bath at 56°C for 30 minutes. Mixing occurs readily and is thorough, provided care is taken that air bubbles do not develop at the interface. The tubes are then supported vertically by means of modelling clay until observations are made. In strong reactions

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agglutination commences almost immediately, while moderate ones take an hour or more. Both strong and moderate reactions can be seen by the naked eye against a dark background with top lighting. Weak, doubtful, and negative reactions are best confirmed by viewing through a hand lens or a stereomicroscope after 24 hours. If observations are to be delayed, tubes should be sealed with collodion to prevent evaporation of the contents.

Test evaluation. The CA test was checked by testing the prepared antigens against 55 undiluted negative control sera obtained from normal persons, 43 of whom had never visited a tropical country; 12 were recent arrivals in Africa from Europe with no history of malaria. For positive controls, serum samples were obtained from 70 local residents who attended the Magila Mission Hospital and could be presumed, on epidemiological grounds, to be regularly exposed to infection with *P. falciparum*.

Of the 70 local residents a group of 28 women had previously participated in a follow-up study and at the time of the examination 14 of them had received a weekly dose of 300 mg of chloroquine base for a period of 5–21 months (average of 12 months). The remainder had received a placebo over a similar period. Monthly blood films from each woman were examined for malaria parasites. No parasites were seen in samples from the protected women whereas patent parasitaemias were confirmed, on at least one occasion, in each of the non-protected women. Two-fold serial dilutions were prepared from these sera and tested against the prepared antigens by means of CA tests. The results were then compared with those from a parallel series of tests carried out by the indirect fluorescent antibody (IFA) test (Voller, 1964; Matola et al., unpublished data), using an homologous antigen.

Immunoglobulin (IgG and IgM) concentrations in the sera of the 28 women from the follow-up study were also determined with the immunodiffusion technique of Mancini, Carbonara & Heremans (1965) using commercially available immunological plates and standards.1

All sera were also tested for cross-reactions with CA antigens made in a similar way from the bovine parasites *Anaplasmia marginale* and *B. bigemina*. Finally a few standard sera from cows recovering from experimental infections with *A. marginale*, *B. bigemina*, and *T. parva* were tested with *P. falciparum* CA antigen. All possible relationships between CA titres, IFA titres, and immunoglobulin concentrations were then subjected to statistical analysis.

Results

Application of the test. The antigen was unaffected when tested by means of CA tests against the 55 undiluted negative control sera derived from people with no history of malaria. On the other hand, distinct clear agglutination of the antigen occurred against the 70 positive control sera (Fig. 1).

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1 Supplied by Hyland Laboratories Inc., Los Angeles, Calif., USA.
Fig. 2. Correlation between CA titres and IFA titres of 70 sera from individuals living in a hyperendemic malaria area.

Group comparisons of CA and IFA titres of the 70 sera from local residents and of immunoglobulin concentrations of the 28 sera from the follow-up groups are given in Tables 1 and 2. CA titres are also compared individually with IFA titres in Fig. 2, IgG levels in Fig. 3, and IgM concentrations in Fig. 4.

A not very strict, though highly significant, correlation exists between the titres obtained by the CA and IFA techniques (Spearman's correlation: $r=0.46$; P two-sided $\approx 0.0005$). No significant correlation was found between CA titres and IgG concentrations ($r=0.30$; P two-sided $\approx 0.13$). The correlation be-

Fig. 3. Correlation between CA titres and IgM concentrations of sera from 28 women participating in a follow-up study. Of these, 14 had received malaria prophylaxis for an average period of 1 year. The 14 others remained non-protected, and revealed malaria parasitaemias during that year on at least one occasion.
between CA titres and IgM concentrations, though also not very strict, can be considered as significant ($r=0.40$; P two-sided $\leq 0.05$).

A comparison of the titres of the sera from 14 protected women with those of the sera from 14 non-protected women revealed that the protected group had significantly lower CA titres (Wilcoxon two-sample test, P two-sided $\leq 0.005$) and IFA titres (P two-sided $\leq 0.05$) than the non-protected group. IgM concentrations were also significantly lower (P two-sided $\leq 0.02$) in the protected group, but there was no significant difference in the IgG concentrations of both groups.

Human sera that reacted negatively with plasmodial antigen were also non-reactive with CA antigens of *A. marginale* and *B. bigemina*. Among the 70 sera that reacted positively with plasmodial antigen, there were a few that also gave positive reactions with the CA antigen of *A. marginale*, but only when undiluted. Of these 70 sera, 55 showed agglutination with the

### Table 1. Comparisons of CA and IFA titres of the 70 sera from local residents of a hyperendemic malaria area

<table>
<thead>
<tr>
<th>Titre class</th>
<th>CA test: no. of sera</th>
<th>IFA test: no. of sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 1/2.5</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>1 : 5/10</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>1 : 20/40</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>1 : 80/160</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>1 : 320/640</td>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>1 : 1 280/2 560</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>total</td>
<td>70</td>
<td>70</td>
</tr>
</tbody>
</table>

### Table 2. Immunoglobulin concentrations of the 28 sera from follow-up groups

<table>
<thead>
<tr>
<th>IgG (mg/100 ml)</th>
<th>No. of sera</th>
<th>IgM (mg/100 ml)</th>
<th>No. of sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\leq 1 000$</td>
<td>2</td>
<td>$\leq 75$</td>
<td>0</td>
</tr>
<tr>
<td>1 001–1 500</td>
<td>5</td>
<td>76–100</td>
<td>3</td>
</tr>
<tr>
<td>1 501–2 000</td>
<td>5</td>
<td>101–150</td>
<td>10</td>
</tr>
<tr>
<td>2 001–2 500</td>
<td>8</td>
<td>151–250</td>
<td>5</td>
</tr>
<tr>
<td>2 501–3 000</td>
<td>6</td>
<td>251–350</td>
<td>6</td>
</tr>
<tr>
<td>$&gt; 3 000$</td>
<td>2</td>
<td>$&gt; 350$</td>
<td>4</td>
</tr>
<tr>
<td>total</td>
<td>28</td>
<td>total</td>
<td>28</td>
</tr>
</tbody>
</table>
B. bigemina CA antigen when undiluted, while 2 sera still showed agglutination with this antigen in dilutions of 1 : 2.5 and 1 : 5.

The sera of some animals recovering from experimental infections with B. bigemina, A. marginale, and T. parva showed positive reactions with P. falciparum CA antigens, but only when undiluted.

Discussion

The preparation of efficient CA antigens from blood protozoa requires, ideally, a prolific source of highly infected material from which parasites can be easily and economically separated, preferably in pure suspension. The concentration of schizonts and trophozoites in the placenta of pregnant women infected with P. falciparum is a well-known phenomenon. As a source of antigen, however, the placenta has some disadvantages since heavy infections are uncommon and cannot be predicted from an examination of blood smears from the mother. Moreover there is a considerable variation in the volume of blood that can be extracted from one placenta and also in the number of lymphocytes and macrophages in the extract that have to be removed, usually at the cost of considerable loss of antigenic material. CA antigens from other primate plasmodia, such as P. knowlesi, P. fieldi, and P. cynomolgi might be used instead of P. falciparum, and their cross-reactivity with CA antibodies to human malaria is worth investigation.

The positive reactions obtained with all sera from a hyperendemic malaria area and the negative reactions with all sera from individuals with no history of malaria suggest that the CA test for malaria has a good basic specificity. The correlation between CA titres and the titres obtained with the accepted IFA technique supports this suggestion.

The correlation between CA titres and IgM concentrations can also be considered as significant. One serum from a hospitalized patient who had an abnormally high IgM concentration of non-specific origin was tested but it failed to agglutinate the malaria CA antigen. Hence, further testing of this antigen with other non-malarious sera of increased IgM concentrations is required. Only then could it be determined whether capillary agglutination in malaria depends on increased IgM levels only, as was the case in the melano-flocculation test of Henry (Trensz & Raab, 1965; Voller, unpublished report to WHO), or would correctly indicate the presence of antiplasmodial antibodies.

The observed cross-reactions of undiluted human malarial sera with CA antigens from bovine intra-erythrocytic parasites, and vice versa, might be explained by the occasional occurrence of heterophile antibodies between phylogenetically different families.

The CA test for malaria might be of value as an indicator for determining the efficacy of malaria control campaigns and it could also find useful application in laboratory experiments.

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