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A COMPARISON OF TRANSMISSION-BLOCKING ACTIVITY WITH REACTIVITY IN A PLASMODIUM FALCIPARUM 48/45-kD MOLECULE-SPECIFIC COMPETITION ENZYME-LINKED IMMUNOSORBENT ASSAY

W. ROEFFEN, T. LENSEN, B. MULDER, K. TEELEN, R. SAUERWEIN, J.
VAN DRUTEN, W. ELING, J. H. E. T. MEUWISSEN, AND P. J. A. BECKERS

Department of Medical Microbiology and Department of Medical Statistics, University of Nijmegen, Nijmegen, The Netherlands

Abstract. Monoclonal antibodies (MAbs) 32F1 and 32F3 react with two independent epitopes of a protein doublet with molecular weights of 48 and 45 kilodaltons (kD) expressed on the surface of Plasmodium falciparum (Pfs48/45) macrogametes and zygotes; only 32F3 blocks transmission. These MAbs were used to develop a Pfs48/45-specific competition enzyme-linked immunosorbent assay (ELISA) using 32F1 to capture antigen and labeled 32F3 for quantification and analysis of the contribution of antibodies in human serum to transmission-blocking activity. A comparison analysis was used to determine agreement of competition ELISA titers and transmission-blocking activity as observed in the bioassay in three groups of serum samples: 37 from European travelers with previous exposure to malaria, 56 from gametocyte carriers, and 66 from schoolchildren from a malaria-endemic area in Cameroon. The index of agreement between outcomes of the ELISA and transmission-blocking assay in gametocyte carriers and in travelers was specifically defined as fair-to-moderate; in schoolchildren the agreement was not significant. The combined analysis of all sera showed a significant and fair-to-moderate agreement between the results of the competition ELISA and the transmission-blocking assay, with a relative specificity of 94% (of 105 cases negative in the transmission-blocking assay, 99 were also negative in the competition ELISA) and a relative sensitivity of 44% (of 54 cases positive in the transmission-blocking assay, 24 were also positive in the competition ELISA). This study shows that a positive C48/45-ELISA is indicative for transmission-blocking activity in the mosquito assay, while a negative result does not exclude transmission-blocking activity.

Gametocytes of Plasmodium falciparum synthesize 230- and 48/45-kilodalton (kD) molecules that remain exposed on the surface of macrogametes and zygotes. Several monoclonal antibodies (MAbs) directed against these molecules block transmission of the parasite to the mosquito vector. One of the targets of transmission-blocking MAbs is the 48/45-kD glycoprotein doublet (Pfs48/45). Two independent, nonrepetitive epitopes of this protein react respectively with MAbs designated 32F1 and 32F3. The presence of 32F3 in a mosquito blood meal blocks transmission of the NF54 isolate of P. falciparum while 32F1 does not. The conventional assay measuring transmission reduction in mosquitoes using a feeder system, as described by Vermeulen and others, is costly, labor intensive, and thus limits the number of sera that can be tested. Development of a serologic test predicting transmission-blocking activity would greatly simplify epidemiologic studies. A positive correlation between antibody reactivity to Pfs230 and transmission-blocking activity has been found using immunoprecipitation to quantify the antibody response. Such a relation was not found for antibody reactivity to Pfs48/45.

This paper describes the development of a two-site competition enzyme-linked immunosorbent assay (ELISA) based on the 32F3 epitope. This competition ELISA was used to analyze several sets of field sera. In addition, the transmission-blocking activity of these field sera was determined using the feeder assay and the results obtained were compared with those of the competition ELISA using a statistical analysis for comparison of methods.

Parasites and Pfs48/45 extract. Mature gametocytes of P. falciparum (isolate NF54) were produced in an automated, large-scale, culture system as described by Ponnudurai and others. Gametogenesis was induced by incubating the mature gametocytes in fetal calf serum (FCS) at room temperature for 30 min. Macrogametes and zygotes were purified as described by Vermeulen and others. Pelleted parasites were stored at −70°C until use. For use in sandwich competition ELISA experiments, Pfs48/45 was extracted in 25 mM Tris-HCl, pH 8.0, containing 0.5% Nonidet P40 (NP40), 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of DNase and RNase. Insoluble debris was pelleted by centrifugation (16,000 × g for 5 min at room temperature) and the soluble protein extract was stored at −70°C. The presence and quantity of Pfs48/45 in the extract was determined in a sandwich ELISA using labeled 32F3 and 32F1.

Transmission-blocking assay. A bioassay with membrane feeders to feed mosquitoes was used to determine transmission-blocking activity. Briefly, while keeping the temperature at 37°C, 14-day-old cultures containing fertile gametocytes were mixed with prewarmed uninfected red blood cells and samples of human sera or MAbs, introduced in prewarmed feeders, and fed to Anopheles gambiae mosquitoes. All samples were tested in two experiments in the presence of complement. The minimal obtainable dilution of the sera was three-fold and the IgG preparations of blocking sera were retested using a 10-fold dilution with the malaria-negative control serum. Fully engorged mosquitoes were separated and held at 26°C. Seven days later, 20 mosquitoes per feeder were dissected and the number of oocysts on the stomach wall was counted and William's mean (adjusted geometric mean) oocysts number was calculated. An experiment was deemed to have succeeded when at least 90% of the mosquitoes examined carried oocysts in each of three controls. The adjusted geometric mean was used to determine the reduction activity (R) according to the equation: R = 

MATERIALS AND METHODS

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60
was performed according to the procedure described by Verhave and others,14 and horseradish peroxidase (HRPO) labeling following the periodate method described by Wilson and Nakane.15 The labeled MAbs were dialyzed against phosphate-buffered saline (PBS) and stored at 4°C.

The basic procedure was that of the two-site ELISA. All incubations were carried out at room temperature. To capture Pfns48/45, wells of microtiter plates (Nunc, Uden, The Netherlands) were coated with 50 μl of a 10 μg/ml concentration of 32F3 in PBS for 45 min. Plates were washed three times with PBS. Wells were saturated with 150 μl of 2% bovine serum albumin in PBS for 30 min. After three washes with PBS, the wells were incubated for 2 hr with 50 μl of the NP40 parasite extract (see above) containing the equivalent of 200,000 gametocytes/zygotes diluted in TNP (25 mM Tris-HCl, pHi 8.0, 150 mM NaCl, 0.1% NP40). Following three washes with PBS, wells were incubated with 30 μl of test serum sample and 30 μl of labeled 32F3 for 2 hr. For the biotin label, an additional incubation step with streptavidin-biotinylated peroxidase complex (1:3,000 diluted in PBS containing 0.05% Tween-20; Amersham Life Sciences, Hertogenbosch, The Netherlands) preceded the incubation with substrate solution. For both labels, the wells were washed four times with PBS and incubated for 20 min with 60 μl of TMB (0.25 mM 3,3',5,5'-tetramethyl benzidine, 0.7 mM H2O2 in 0.1 M sodium acetate, pH 5.5) substrate solution. The peroxidase substrate reaction was stopped by adding 60 μl of 2 M H2SO4 and the optical density (OD) was determined in an ELISA reader at 450 nm (Titertek Multiskan MCC/340; ICN, Zoetermeer, The Netherlands).

For optimal immunoreactions, labeled MAbs and antigen were tested in a checkerboard titration. From this antigen-antibody titration, concentrations of labeled MAb and antigen were chosen resulting in > 60% of the maximal OD of labeled MAb and an OD reading > 1.0.

Comparison of the transmission-blocking assay and competition ELISA. The results of the competition ELISA were compared with those obtained by the transmission-blocking assay and analyzed by a statistical test on agreement using a calculated index of observed agreement.5-7 The index kappa (K) was calculated from the observed and expected (by chance) frequencies on the diagonal of the 2 × 2 table of transmission-blocking activity outcomes in the bioassay (≥ 85%, < 85%) and results of the competition ELISA (positive, negative). The index is given by K = (P0 - Pc)/1 - Pc, where

<table>
<thead>
<tr>
<th>Value of K</th>
<th>Strength of agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤0.20</td>
<td>Poor</td>
</tr>
<tr>
<td>0.21-0.40</td>
<td>Fair</td>
</tr>
<tr>
<td>0.41-0.60</td>
<td>Moderate</td>
</tr>
<tr>
<td>0.61-0.80</td>
<td>Good</td>
</tr>
<tr>
<td>0.81-1.00</td>
<td>Very good</td>
</tr>
</tbody>
</table>

The approximate error of K is SE(K) = \(\sqrt{n(1 - P_c)/2}\) and the approximate 95% confidence interval is given by K ± 1.96 SE(K).

The index of agreement K has a maximum of 1.00 when agreement is perfect; values of zero or less indicate no agreement better than that due to chance. While no absolute interpretation is possible, the guideline proposed by Altman6 was used to determine the strength of agreement (Table 1).

**RESULTS**

**Testing of the labels and competition with serum antibodies.** The optimal concentration of labeled MAb and the optimal amount of antigen coated per well as determined in the checkerboard titration were 2 μg/ml for the biotin label and 200,000 macrogamete/zygote equivalents/well, and 1 μg/ml for the HRPO label and 100,000 macrogamete/zygote equivalents/well, respectively. The maximal OD of the
Two-site competition enzyme-linked immunosorbent assay. Monoclonal antibody (MAb) 32F1 was used to capture Pfs48/45 from an NP-40 extract. + = horseradish peroxidase (HRPO)-32F3 (1 μg/ml with 100,000 macrogamete/zygote equivalents/well) and ▲ = biotin-32F3 (2 μg/ml with 200,000 macrogamete/zygote equivalents/well) were used as labels in competition with serial dilutions of unlabeled 32F3. The horizontal dotted line represents the 50% competition value and the vertical dotted lines represent the concentration of unlabeled MAb needed to arrive at a 50% competition with the labeled MAb.

HRPO-32F3 label was higher than that of the biotin-32F3 label using the same concentration of antigen. Competition of labeled MAb with increasing concentrations of unlabeled MAb is depicted in Figure 1. The 50% competition values of the corresponding unlabeled MAb were 0.25 μg/ml for the 1.0 μg/ml HRPO label and 1.3 μg/ml for the 2.0 μg/ml biotin label. In addition, it appeared that the biotin-32F3 competition ELISA was positive, with seven of 19 sera having positive transmission-blocking activity as observed in the bioassay, whereas the HRPO-32F3 competition was positive for nine of 19 sera. Due to the limited sensitivity of the biotin-32F3 competition ELISA, human sera could not be diluted more than 1:10, preventing a complete titration of the sera. The MAb 32F1 (anti-Pfs48/45) and MAbs against the 25-kD and 230-kD antigens were negative in the competition test up to a concentration of 330 μg/ml.

Figure 2 shows examples of the competition of serial dilutions of sera with the HRPO-32F3 label. Data of the negative serum are the mean of 14 individual sera tested on three separate occasions. Calculation of the overall intra-assay variation resulted in an average deviation of the mean OD of 0.08 (10%). The overall interassay variation resulted in an average deviation of the mean OD of 0.11 (14%). The titration values of three sera that were positive in the competition ELISA are depicted using the overall mean of duplicate determinations of two independent tests.

Control experiments with 14 sera from Dutch blood bank donors exhibited a nonspecific reduction up to a maximum of 15% of the OD of the HRPO-32F3 label compared with reactions without competing serum antibodies. This nonspecific reduction was not seen with IgG preparations of these sera. Nevertheless, the competition titer found for sera and their corresponding IgG preparations were the same.

Comparison of competition ELISA and transmission-
Comparison of activity in the transmission-blocking (TB) assay and in the Pf48/45 competition enzyme-linked immunosorbent assay (C48/45-ELISA) in all field sera combined

<table>
<thead>
<tr>
<th>TB assay</th>
<th>C48/45-ELISA results</th>
<th>No. positive</th>
<th>No. negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥85%</td>
<td>24</td>
<td>30</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>&lt;85%</td>
<td>6</td>
<td>99</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>129</td>
<td>159</td>
<td></td>
</tr>
</tbody>
</table>

* Relative specificity = 94% (99 or 105); relative sensitivity = 44% (24 or 54); index of agreement K = 0.44.

Transmission-blocking assay in several groups of sera. Table 2 shows the results of the competition ELISA and the transmission-blocking assay for the sera of gametocyte carriers, travelers, and schoolchildren. Using the HRPO-32F3 label, 17 (30%) of 56 of the gametocyte carriers exhibited significant competition in the ELISA. These results were compared with the corresponding transmission-blocking activity observed in the bioassay. By shifting the transmission-blocking assay cutoff point in the gametocyte carrier group in the range of 10% to 90%, it appeared that the maximum degree of agreement between the transmission-blocking assay and the C48/45-ELISA was at the 85% reduction point. Furthermore, it appeared that the agreement between the transmission-blocking assay and the C48/45-ELISA was not greater than chance if the cutoff point of the transmission-blocking assay was set at a reduction lower than 70% (95% confidence interval of K included the value 0). At the 85% reduction point, the index K assumed the value 0.40, indicating fair-to-moderate agreement between the outcomes of the competition ELISA and the transmission-blocking assay (Table 3). Four of the 17 positive sera from gametocyte carriers in the C48/45-ELISA test had oocyst counts that did not meet the 85% reduction standard. Of the 25 sera that were positive in the transmission-blocking assay, 13 had antibodies competing with the HRPO-32F3 MAb, indicating a relative sensitivity of 52%. Of 31 sera considered negative by the transmission-blocking assay, 27 were also found to be negative by the C48/45-ELISA, showing a relative specificity of 87% for the competition ELISA.

Nine (24%) of 37 travelers exhibited significant activity in the ELISA. Nine (47%) of 19 sera positive in the transmission-blocking assay had antibodies competing with the HRPO-32F3 MAb. All 18 sera considered negative by the transmission-blocking assay were also negative by the C48/45-ELISA. The index of agreement K between the transmission-blocking assay and the C48/45-ELISA for the group of travelers assumed a value of 0.47, indicating moderate agreement (Table 3).

Table 3

<table>
<thead>
<tr>
<th>Group</th>
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<th>95% confidence interval</th>
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<tr>
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<td>Fair to moderate</td>
</tr>
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<td>0.19–0.75</td>
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</tr>
<tr>
<td>Schoolchildren</td>
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<td>0.22</td>
<td>−0.23–0.67</td>
<td>Not significant</td>
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</table>

DISCUSSION

An MAb-based competition ELISA was developed to study the natural antibody reactivity to Pf48/45 in field sera in comparison with transmission-blocking activity as observed in a bioassay. The 32F3-labeled MAb was used in this competition ELISA because of its blocking activity in the transmission-blocking assay. Competition of antibodies in field sera with labeled 32F3 for binding to Pf48/45 might therefore be relevant for transmission-blocking activity. Transmission blockade has not been found in serum of rabbits immunized with recombinant Pf48/45 (Roefen W and others, unpublished data). Therefore, Pf48/45 extract from parasites was used as antigen in the competition ELISA.

The concentration of unlabeled antibody necessary to reduce binding of the labeled MAb in the competition ELISA was lower for HRPO-labeled 32F3 than for biotin-labeled 32F3. This was supported by the observation that in the competition ELISA with HRPO-32F3 as label, none of 19 blocking field sera were positive in contrast to seven of 19 when biotin-32F3 was used. Therefore, HRPO-labeled 32F3

Table 4

Comparison of activity in the transmission-blocking (TB) assay and in the Pf48/45 competition enzyme-linked immunosorbent assay (C48/45-ELISA) in all field sera combined

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Comparison of activity by index of agreement K in the Pf48/45 competition enzyme-linked immunosorbent assay (C48/45-ELISA) and the transmission-blocking assay in sera of gametocyte carriers, travelers, and schoolchildren

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positive in 44% of the sera with transmission-blocking activity from a radiolabeled gametocyte extract or correlate with either the capacity of these field sera to pre- 

ticipation ELISA. The results of this competition ELISA did not correlate with the activity of the test and/or to define additional transmission-blocking targets.

Acknowledgments: We are grateful to Truus Derks and Marianne Sieben for the production and purification of monoclonal antibodies, to Theo Arens and Tita Oettinger for serologic testing, to Marga Bolmer, Anrienne Huisman, and Gerjan van Gemert for parasite cultures and transmission experiments, and to Christina Celluzzi for critical reading of the manuscript.

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Authors’ addresses: W. Roeffen, T. Lens, B. Mulder, K. Teelen, R. Sauerwein, W. Eling, J. H. E. T. Meuwissen, and P. A. J. A. Beckers, Department of Medical Microbiology, University of Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands. J. Van Druten, Department of Medical Statistics (MSA), University of Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands.

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