Brief Definitive Report

Transmission Blocking Immunity in *Plasmodium vivax* Malaria: Antibodies Raised against a Peptide Block Parasite Development in the Mosquito Vector

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Summary

One approach towards the development of a vaccine against malaria is to immunize against the parasite sexual stages that mediate transmission of the parasite from man to mosquito. Antibodies against these stages, ingested with the blood meal, inhibit the parasite development in the mosquito vector, constituting "transmission blocking immunity." Most epitopes involved in transmission-blocking immunity depend on the tertiary conformational structure of surface antigens. However, one of the transmission-blocking monoclonal antibodies we have raised against *Plasmodium vivax* reacts with a linear epitope on both asexual stages and gametes. This monoclonal antibody (A12) is capable of totally blocking development of the parasite in the mosquito host when tested in membrane feeding assays with gametocytes from *P. vivax*–infected patients. Immune screening of a *P. vivax* λgt11 genomic expression library with A12 led to the isolation of a clone to which was mapped the six-amino acid epitope recognized by A12. Antisera raised in mice against a 12-mer synthetic peptide containing this epitope coupled to bovine serum albumin not only had high titers of antipeptide antibodies as measured by enzyme-linked immunosorbent assay, but in addition recognized the same 24- and 57-kD parasite components as A12 on Western blots and reacted with the parasite by immunofluorescence. When tested in membrane feeding assays, these antibodies have significant suppressive effects on parasite development in the mosquito.

Transmission-blocking vaccines against malaria are based on immunity mediated by antibodies against parasite sexual stages. These antibodies block the development of the malaria parasite in the mosquito host (reviewed in reference 1). Such vaccines, while not protecting the vaccinated individual, would prevent subsequent transmission of the parasite. They would thus be effective at a community level, both by reducing transmission and also by preventing the spread of parasites rendered resistant through drug or immune pressure exerted on asexual blood stages. It has been demonstrated that *Plasmodium vivax* patients can readily develop antibodies against parasite sexual stages (2). This leads to natural transmission-blocking immunity that significantly lowers the incidence of infection (3). The ability of antibodies to block parasite transmission or suppress infectivity is tested by membrane feeding, a biological assay that directly reflects the phenomenon arising in naturally infected patients (described in reference 4).

mAbs with transmission-blocking activity have allowed the definition of specific targets of transmission-blocking immunity on the surface of *P. falciparum* sexual stages (5, 6). Several genes coding for these target antigens have now been cloned (Pfs25, [7] Pfs48/45 [8], and Pfs230 [9]). Such cloning was achieved through screening libraries with oligonucleotide probes, the sequence of which was derived from microsequencing of purified parasite antigens, not through immune screening of expression DNA libraries. This is because transmission-blocking antibodies predominantly recognize conformationally dependent epitopes that are not usually suitably reproduced in *Escherichia coli* (1).

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Because of the lack of significant cross-reactive immunity and of DNA cross-hybridization between *P. falciparum* and *P. vivax*, the characterization of species-specific molecules which are targets of transmission-blocking immunity, is required for the development of an effective vivax transmission–blocking vaccine. We have characterized such molecules through isolation of several transmission-blocking mAbs (described in reference 10). All but one of these antibodies recognize epitopes that depend on tertiary structures of the parasite surface antigens and are thus not good candidates for screening expression libraries constructed in *E. coli.* However, the difficulties inherent in obtaining large quantities of *P. vivax* parasites (this parasite cannot be maintained in long-term cultures), limit the use of protein purification and micro-sequencing as the basis for cloning relevant genes.

Here we describe the use of a *P. vivax* transmission-blocking mAb (A12), which recognizes a linear epitope, to identify a clone upon immune-screening of a λgt11 *P. vivax* expression library. The six amino acids which encompass the linear epitope recognized by this mAb have been mapped within the translated amino acid sequence of this clone and the ability of a corresponding peptide assessed for its ability to elicit transmission-blocking or infectivity-suppressing antibodies. Antibodies raised in mice against a synthetic peptide comprising this epitope have the capacity to significantly suppress infectivity of the parasite in the mosquito, as measured by membrane feeding assays. Both the monoclonal and monospecific polyclonal antibodies recognize the same parasite components on Western blots and react with sexual as well as asexual stage parasites by immunofluorescence.

Materials and Methods

Parasite Material. *P. vivax* gametocytes for membrane feeding experiments and Western blots were obtained, under informed consent, from patients admitted to the Colombo General Hospital in Sri Lanka. Female gametes were prepared as described in Munesinghe et al. (4). Parasites used for all nucleic acid studies were obtained from *Saimiri sciureus* monkeys (Guyana phenotype) infected with the *P. vivax* Belem isolate. Purified parasites were prepared and extracted for DNA as detailed in (11), for RNA by the method of Chomczynski and Sacchi (12) or were resuspended in nonreducing SDS-PAGE sample buffer for Western blots (13). Immunofluorescence slides were prepared from infected human or monkey blood. Immunofluorescence on air-dried slides was performed as described (13) with the modification of 5% FCS added to incubation solutions.

mAb. The mAb A12 was raised against the surface of *P. vivax* asexual stage parasites. It has previously been described (13).

Library Production and Screening. The *P. vivax* genomic DNA library, has been previously described (11). Both the λgt11 and pGex libraries were constructed through DNase1 digestion of DNA in the presence of manganese, based on the method of Mattei et al. (14). Antibody screening was carried out on 10^5 phage from the unamplified λgt11 library or 5,000 pGex colonies, on appropriate filters containing 10 mM IPTG (isopropyl-β-D-thiogalactoside), using the A12 mAb ascitic fluid and iodinated protein A (Amersham France SA, Les Ulis, France). Sequencing was carried out according to dideoxy technology using the Sequenase kit (United States Biochemical Corp., Cleveland, OH) as recommended by the manufacturers. Other DNA techniques were carried out based on standard protocols of (15).

SDS-PAGE and Western Blot Analysis. Approximately 2×10^7 parasites were loaded onto 10% mini SDS-PAGE gels and migrated under reduced conditions, using prestained molecular weight markers (Bio-Rad Laboratories, Hercules, CA). Western blots were carried out in a Bio-Rad Mini Trans-Blot Cell. Antibodies were diluted appropriately and reacted with alkaline phosphatase–conjugated anti-mouse second antibody (diluted 1:7,500) and appropriate substrates (Promega, Madison, WI).

Peptide Synthesis. A set of 46 peptides was purchased from Cambridge Research Biochemicals Ltd. (Northwich, Cheshire, UK). Each peptide consisted of 12 residues overlapping by one amino acid, bound to a solid polyethylene pin support in the same layout as a standard ELISA plate. The mAb culture supernatant was reacted against these pins and revealed using an antimouse (Promega) conjugate diluted 1:2,000. This was carried out according to the manufacturer's recommendations, except that the antibody and conjugate were diluted in 1% vol/vol FCS, 0.1% vol/vol Tween-20, 0.1% wt/vol BSA in 0.01 M PBS, pH 7.2, instead of the solution given in the protocol. Free synthetic peptides (>95% pure by HPLC) were produced at the Pasteur Institute Organic Chemistry Unit, using solid-phase technology containing a cysteine residue for conjugation to activated BSA using the Imject activated Super-Carrier system (Pierce, Rockford, IL).

Immunization Protocols. 6–8-wk-old female BALB/c mice (Iffa-Credo, L'Arbresle, France) were used throughout. Peptide constructs were used to immunize 10 mice per group, with 25 μg i.p. of conjugated peptide-BSA in a total volume of 100 μl per mouse using aluminium hydroxide (Pierce) as adjuvant (final concentration 2 mg/100 μl). Fusion proteins were used to immunize five mice per group, with 50 μg i.m. per mouse using Freund's complete and incomplete adjuvant. After initial immunization, mice were boosted every 3 wk and bled 7 d after boosting.

ELISA. Antibody titers were tested for individual mice after every immunization with the peptide conjugates, by ELISA either employing free peptide as antigen or appropriate peptide coupled to KLH as carrier. 96-well plates (Nunc, Roskilde, Denmark) were routinely coated at 0.5 μg/ml quenched in PBS-Tween-gelatin and revealed using horseradish peroxidase anti-mouse conjugate (Promega) diluted 1:2,000. Plates were read at 405 nm (490 value subtracted automatically) via a plate reader (Molecular Devices; Palo Alto, CA) and corresponding software.

Membrane Feeding Assays. Each assay was carried out using gametocyte-infected blood from a *P. vivax* patient. For each assay, 150 μl of infected erythrocytes was resuspended in 75 μl of mouse antipeptide sera and 75 μl of normal AB human sera. This was fed to 50 female *Anopheles tessellatus* mosquitoes in a membrane-feeding apparatus prewarmed to 37°C. Nonfed mosquitoes were subsequently removed and the fed mosquitoes maintained at 26°C ± 2°C. The number of oocysts developing in the mid gut was examined microscopically after 7 d (4).

Results and Discussion

Upon the immune-screening of a λgt11 *P. vivax* genomic expression library with the A12 mAb, a clone was identified which contained a 1.5-kb insert. Upon induction, a recombinant fusion protein was produced in *E. coli* corresponding to 132 kD on reduced SDS-PAGE gels, indicating a parasite component of ~16 kD. The translated amino acid sequence bore no significant homology to sequences in the GenBank

database (release 82), using a BLAST search (16) (data not shown).

To identify the linear epitope of this *P. vivax* transmission-blocking mAb, a sublibrary corresponding to the λgt11 clone was constructed in a bacterial expression system. DNase1-generated fragments were prepared according to previous protocols (14), EcoRI linkered and ligated into the pGex expression vector (17). These clones were then screened with the A12 mAb. Out of 25 putative clones, 23 constructs expressing the A12 epitope were confirmed by Western blot analysis. Corresponding DNA sequences were obtained using plasmid-specific oligonucleotide primers, and ranged between 96 and 402 bp, all clones overlapping with 100 bp at the 3′ end of the coding sequence.

One of these constructs (clone 21, Fig. 1) was used to immunize BALB/c mice and the sera obtained reacted on Western blots of either *P. vivax* asexual stages or gametocytes extracted under reducing conditions. The antibodies produced upon immunization with the fusion protein identified the same pattern of bands as the original mAb A12, corresponding to a more intense 24-kD band plus an additional band of 57 kD, as estimated from reduced SDS-PAGE gels, (Fig. 2 *A*, panels *1* and *2*).

From an analysis of the DNA sequence encoding the open reading frame in phase with the Glutathione S-Transferase (GST) fusion protein of the smallest of the pGex clones, (clone 21, Fig. 1 *B*), the A12 epitope was mapped to a 32-amino acid region, corresponding to the COOH-terminal region of the predicted protein. To further delimit the A12 epitope sequence, synthetic peptide pin technology was employed. 46 overlapping peptides corresponding to this region were constructed and incubated with the A12 mAb. This mapping revealed the epitope of the monoclonal to consist of the six amino acids: TWEVLH (Fig. 1 *A*). A 12-mer peptide (Ep: CPLPTWEVLHDGS), corresponding to the predicted amino acid sequence containing this epitope was synthesized. As a control, a peptide representing the "Universal T cell epitope" from the *P. falciparum* CS protein, (Tu: EKKIAKMEKASSVFNVVCG) (18) was employed. Peptides were coupled to a BSA carrier before immunization of BALB/c mice using aluminium hydroxide (Alum) as adjuvant, the only adjuvant currently accepted for human use.

Upon two immunizations with the Ep peptide, antibodies were produced which reacted with the same pattern on parasite Western blots as the A12 mAb (Fig. 2 *A*, lanes *1* and *3*). The presence of a higher molecular weight band (~65 kD) on Western blots when both the epitope peptide and control sera are reacted with the gamete antigen is probably due to the presence of human serum albumin contaminating this antigen preparation. The fact that the profiles on Western blotting are otherwise identical indicates that the target molecule recognized by these antibodies may indeed be present in both asexual as well as sexual blood stage parasites, unlike other targets of transmission-blocking immunity described to date (1). A12 and antipeptide sera demonstrated reactivity with the parasite, both with asexual parasites and with female gametes on immunofluorescence. There was no reactivity with the control peptide sera at the dilutions tested (1/20–1/500).

Each mouse serum sample was tested for reactivity individually by ELISA then pooled for use in transmission-blocking assays. Individual mice ELISA titers are not shown but were highly uniform throughout each group of mice. The antiepitope titers for the pooled sera used in membrane-feeding analysis are shown in Fig. 3. Antibody titers specific to both peptides reached high levels even after the second immunization

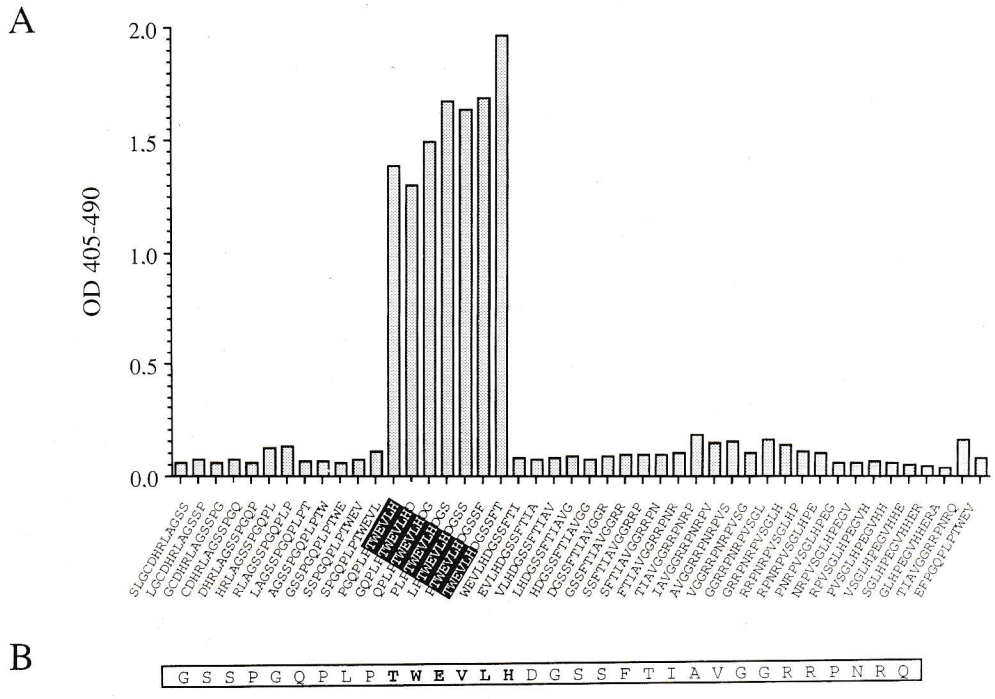

Figure 1. (*A*) Mapping of the transmission-blocking mAb A12 epitope using overlapping peptides derived from the pGex expression clone 21. The 46 peptides, each overlapping by one residue, are shown on the *x* abscissa plotted against the absorption value on reaction with the mAb. The deduced epitope (TWEVLH) is highlighted in the black box. (*B*) The translated amino acid sequence of clone 21, the smallest DNA fragment obtained able to encode a fusion protein recognized by the A12 mAb, the epitope shown in boldface. The complete sequence data are available from EMBL/GenBank/DDBJ under accession number X82416.

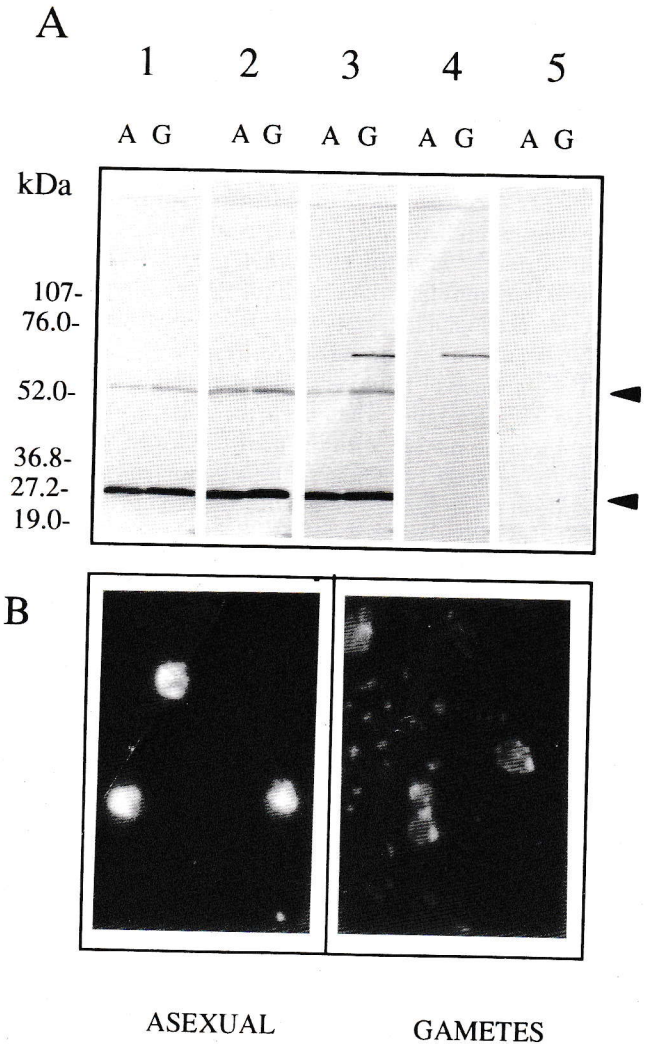

Figure 2. (*A*) Western blots of reduced *P. vivax* antigen from either asexual (*A*) or gamete (*G*) preparations, reacted with the A12 mAb in lane *1*, the pGex clone 21 recombinant sera (third immunization) in lane *2*, and the epitope peptide antibodies in lane *3*, consisting of the antibody pool employed in membrane feeding, fifth immunization. Control antisera raised against the control peptide and pGex GST fusion protein alone are illustrated in lanes *4* and *5*, respectively. All sera were employed diluted 1/400. (*B*) Immunofluorescence of air-dried parasite antigen from asexual stages and gamete preparation reacted with the epitope peptide antibodies. Sera was diluted 1/250; the control peptide antisera gave no fluorescence at this dilution and is not shown.

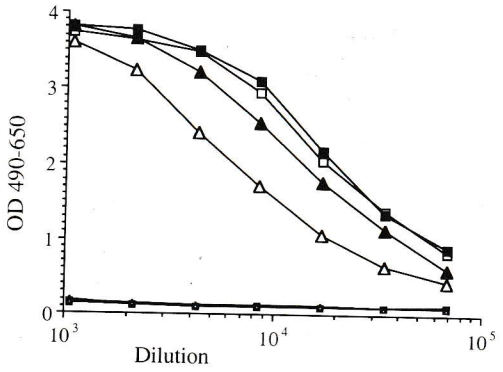

Figure 3. ELISA titers of pooled sera used in membrane-feeding assays from the third and fifth immunizations. The uncoupled peptide at 3 μg/ml was used as antigen. Plates were coated and serum incubated overnight at 4°C before second antibody incubation at 37°C for 3 h. Ep indicates the A12 epitope sera and Tu, the control sera. (□) Ep third immunization; (▲) Ep fifth immunization bled after 7 d; (■) Ep fifth immunization bled after 22 d; and (△) Ep fifth immunization bled 44 d after immunization. All the Tu sera give very low absorption (<0.2 $OD_{405-490}$ U) when tested against the Ep peptide. The reciprocal of the dilution is shown on the *x* abscissa (*Dilution*).

(data not shown) and subsequent boosts did not significantly increase these titers.

The antipeptide sera were tested in 10 membrane-feeding experiments carried out using gametocyte-infected blood from eight different *P. vivax* patients and the results are presented in Table 1. Feedings were carried out using sera obtained after either three or five immunizations as detailed in the table legend. In 8 out of 10 experiments, the antisera suppressed infectivity of *P. vivax* gametocytes to a statistically significant extent, as judged by the number of parasites (oocysts) developing in the mosquito, the infectivity being reduced to between 6 and 33% of the control. Antiserum obtained at three different time points after the fifth injection were tested against the same parasite isolate (experiments 8–10) and significant infectivity suppressive effects were shown even 6 wk after immunization. Despite the variability of these results, they represent a significant reduction in infectivity. In 5 out of the 10 experiments, the immune sera reduced the number of mosquitoes infected to ⩽50% of that in controls and in every case there is a reduction in the number of mosquitoes infected for the epitope peptide compared to the control (after correcting for the numbers of mosquitoes counted).

The variability in the infectivity suppressive effects demonstrated in these experiments may be explained by the fact that each experiment was performed using gametocyte-infected blood from a different infected donor. They are therefore likely to differ in the gametocyte count, the ratio of male and female gametocytes, and possibly in recognition of the epitope, all of which are known to influence the impact of an infectivity suppressive antibody. The inability to use a standard parasite isolate for testing is an invariable technical difficulty encountered with *P. vivax* due to the lack of continuous *in vitro* culture.

It has recently been proposed that control measures aimed at decreasing transmission rates of malaria parasites may be more effective than previously predicted (19). Moreover, the observation that a reduction of the levels of transmission through the use of insecticide treated bed nets could significantly reduce morbidity (20, 21) indicates that transmission-blocking immunity, even if incomplete, could have an impact on disease as well as reduce the spread of drug- or vaccine-resistant parasites. We have thus demonstrated that antibodies which suppress infectivity can be raised in mice by immunization with a small peptide. This raises the hope that at least in the case of some target antigens of transmission-blocking immunity, eukaryotic expression of large recom-

Table 1. *Membrane Feeding Results Demonstrating the Effect of the Anti-A12 Epitope Peptide Sera on P. vivax Infectivity to Mosquitoes*

Expt. No.	MR No.	Mean oocyst per gut Anti-sera raised against: Epitope peptide	Control peptide	Percent infectivity Mann-Whitney U test p value at 95%
1‡	3324	1.6 (8/20)	27.3 (12/20)	5.8% $^*p = 0.0179$
2‡	3331	12.0 (8/20)	53.1 (19/22)	22.6% $^*p = 0.0003$
3‡	3334	16.3 (11/21)	88.9 (11/13)	18.3% $^*p = 0.0008$
4‡	3339	20.8 (8/18)	62.5 (9/17)	33.3% $p = 0.3141$
5‡	3340	23.5 (10/17)	94.4 (20/21)	24.8% $^*p = 0.0008$
6‖	3393	3.3 (10/17)	31.3 (11/20)	10.5% $p = 0.0783$
7‖	3396	28.8 (7/20)	94.5 (13/21)	30.5% $^*p = 0.0183$
8§	3398	14.6 (5/22)	56.8 (14/22)	24.7% $^*p = 0.0192$
9‖	3398	11.4 (5/21)	51.3 (13/20)	22.2% $^*p = 0.0032$
10¶	3398	18.5 (6/20)	57.9 (14/20)	31.4% $^*p = 0.0155$

The percent infectivity of the A12 epitope peptide (Ep) compared to the control (Tu) is shown for each of the 10 feedings (Expt. No.) where p values correspond to a 95% confidence level for a Mann-Whitney U test, applied to random samples. The MR No. is the parasite isolate number used in each feeding. The number of mosquitoes infected/number mosquitoes dissected is shown in parentheses for the Ep and Tu peptide serum. The mean oocyst per gut is shown after feeding mosquitoes in the presence of antibodies raised against either the epitope or control peptide.
* $p < 0.05$ is significant.
‡ Sera from the third immunization.
§ Sera from the fifth injection taken 7 d after immunization.
‖ Sera from the fifth injection taken 22 d after immunization.
¶ Sera from the fifth injection taken 44 d after immunization.

binant molecules reproducing specific tertiary structures may not be essential.

Immunization of mice with the A12 epitope peptide led to antibodies that completely blocked parasite development in a number of mosquitoes, and significantly lowered oocyst burdens using experimental infections of laboratory mosquitoes. Similar results, if they were to be obtained in natural infections, where oocyst numbers are usually lower than those obtained here, could have significant effects on *P. vivax* incidence and morbidity.

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