An Anti-Chitinase Malaria Transmission–Blocking Single-Chain Antibody as an Effector Molecule for Creating a *Plasmodium falciparum*–Refractory Mosquito

Fengwu Li, Kailash P. Patra, and Joseph M. Vinetz

Division of Infectious Diseases, Department of Medicine, University of California–San Diego School of Medicine, La Jolla

Indirect evidence has suggested the existence of a second chitinase gene, *PgCHT2*, in the avian malaria parasite *Plasmodium gallinaceum*. We have now identified *PgCHT2* as the orthologue of the *P. falciparum* chitinase gene *PfCHT1*, a malaria transmission–blocking target. Computational phylogenetic evidence and biochemical and cell biological functional data support the hypothesis that an avian-related *Plasmodium* species was the ancestor of both *P. falciparum* and *P. reichenowi*, and this single lineage gave rise to another lineage of malaria parasites, including *P. vivax*, *P. knowlesi*, *P. berghei*, *P. yoelii*, and *P. chabaudi*. A recombinant PfCHT1/PgCHT2-neutralizing single-chain antibody significantly reduced *P. falciparum* and *P. gallinaceum* parasite transmission to mosquitoes. This single-chain antibody is the first anti-*P. falciparum* effector molecule to be validated for making a malaria transmission–refractory transgenic *Anopheles* species mosquito. *P. gallinaceum* is a relevant animal model that facilitates a mechanistic understanding of *P. falciparum* invasion of the mosquito midgut.

Malaria is the most important parasitic disease of humans: hundreds of millions of people are infected annually, resulting in enormous morbidity and an estimated 1–3 million deaths. Malaria transmission begins when a mosquito injects infectious sporozoites into a vertebrate host while ingesting a blood meal, and it continues when a mosquito ingests a gametocyte-containing blood meal. A complex developmental process ensues in the mosquito midgut after ingestion of a blood meal. Male and female gametes merge to form zygotes that elongate into the invasive motile form, the ookinete. The ookinete must traverse the chitin-containing peritrophic matrix surrounding the ingested blood meal en route to invading the midgut epithelium to become a sporozoite-forming oocyst [1]. The ookinete secretes a family 18 chitinase [2–4] that facilitates parasite penetration and traversal of the peritrophic matrix, as has been shown in gene knockout studies [5, 6]; membrane feeding assays with allosamidin, which is a family 18 chitinase inhibitor [7]; and membrane feeding assays with chitinase-specific antibodies [8, 9]. A monoclonal antibody (MAb) raised against the recombinant *P. falciparum* chitinase PfCHT1, designated 1C3, neutralizes PfCHT1 enzymatic activity, recognizes a presumptive non-*PgCHT1* chitinase in the avian malaria parasite *P. gallinaceum*, and significantly reduces the infectivity of both *P. falciparum* and *P. gallinaceum* in mosquitoes [8, 9]. The *P. gallinaceum* transmission–blocking effect of 1C3, the delineation of a novel secretion-associated structure in the apical end of the *P. gallinaceum* ookinete, and Western immunoblotting [8] suggested that a second PfCHT1-related chitinase gene, provisionally designated *PgCHT2*, existed in the avian malaria parasite [3, 8, 10]. Given the malaria transmission–blocking properties of 1C3 and its recognition of both PfCHT1 in *P. falciparum* ookinetes and PgCHT2 in *P. gallinaceum* ookinetes, we performed the present study to...
Figure 1. Identification of the Plasmodium gallinaceum and P. reichenowi orthologues of the P. falciparum chitinase PfCHT1 and alignment of chitinases. The PfCHT1 epitope recognized by the chitinase-neutralizing monoclonal antibody 1C3 [8] is indicated. The Signal P algorithm was used to predict the eukaryotic secretory signal sequences, as indicated by underlining [23]. The low complexity region, catalytic domain, substrate-binding site, catalytic site, and putative chitin-binding domain are indicated. An asterisk indicates identical amino acids in all species, a colon indicates strong conservation, and a period indicates weak conservation. GenBank accession nos. for the chitinases are as follows: P. falciparum PfCHT1 (AF172445), P. gallinaceum PgCHT2 (AY842482), P. reichenowi PrCHT1 (AY842483), P. gallinaceum PgCHT1 (AF064079), P. berghei PbCHT1 (AJ305256), P. yoelii PyCHT1 (AB106898), P. chabaudi PchT1 (AB106897), P. vivax PvCHT1 (AB106896), and P. knowlesi PkCHT1 (AB106867).
Figure 2. Relationship of PgCHT2, the Plasmodium gallinaceum orthologue of the P. falciparum chitinase PfCHT1, to other chitinases. A, Schematic representation of the short forms of chitinase found in P. falciparum, P. gallinaceum, and P. reichenowi. These short forms of chitinase lack proenzyme and putative chitin-binding domains, compared with the long forms of chitinase found in P. gallinaceum, the primate malaria parasites P. vivax and P. knowlesi, and the rodent malaria parasites P. berghei, P. yoelii, and P. chabaudi, which contain proenzyme and putative chitin-binding domains in addition to the substrate-binding and catalytic active sites. B, Unrooted phylogenetic tree. The close relationship between PfCHT1, PgCHT2, and PrCHT1, the chitinase of the chimpanzee malaria parasite P. reichenowi, are indicated. These are distant from PgCHT1, the other chitinase of P. gallinaceum; PbCHT1, PyCHT1, and PchCHT1, the chitinases of the rodent malaria parasites; PvCHT1, the chitinase of P. vivax; and PkCHT1, the chitinase of the primate malaria parasite P. knowlesi. The tree was constructed using the CLUSTALW program and the neighbor-joining method [24, 25]. The tree was drawn using TreeView [26]. The no. of amino-acid substitutions per site of 0.1 is indicated. Sequence data for PgCHT2 and PrCHT1 were obtained from the Sanger Centre P. gallinaceum and P. reichenowi sequencing projects; the DNA sequence for PgCHT2 was independently verified by sequencing of a cDNA clone. Sp, signal peptide.

Determine whether PgCHT2 encodes the orthologue of PfCHT1, and, if so, whether a recombinant single-chain antibody derived from the 1C3 hybridoma would reduce P. falciparum infectivity in Anopheles species mosquitoes and P. gallinaceum infectivity in Aedes aegypti mosquitoes. The characteristics of PgCHT2 and the presence of 2 chitinase genes in P. gallinaceum suggest that an avian malaria parasite was the ancestor of both rodent and primate lineages of Plasmodium species and gave rise to the chitinase of P. falciparum and the closely related chimpanzee malaria parasite P. reichenowi. The recognition of both PfCHT1 and PgCHT2 by the same recombinant single-chain antibody validates P. gallinaceum as a model system for understanding the mechanistic role that these chitinases play in ookinete invasion of the mosquito midgut. Furthermore, these data provide evidence of a new effector gene that has potential utility for the generation of malaria transmission–refractory transgenic mosquitoes and provides insight into the mechanism of anti-chitinase malaria transmission–blocking antibodies.

Materials and Methods

Parasites, mosquitoes, and cell lines. P. gallinaceum strain 8a was maintained by cyclical passage through chickens and Aedes aegypti mosquitoes. P. falciparum strain 3D7 was maintained in vitro in continuous cultivation. The anti–PfCHT1 MAb–producing hybridoma clone 1C3 has been described elsewhere [4]. The animal experimentation was approved by the University of California–San Diego Institutional Animal Care and Use Committee and followed US federal guidelines.

Assembly and recombinant expression of the 1C3 ScFv gene. mRNA was extracted from 5 × 10^7 1C3 hybridoma cells using the QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech). By use of the Mouse ScFv Module/Recombinant Phage Antibody System (Amersham Pharmacia Biotech), the first-strand cDNA was synthesized from 1C3 mRNA by reverse transcription using random hexamer priming. The 1C3 heavy variable (VH) and light variable (VL) segments were separately
MKFLNVALVFVMYYYYIAGMPMAQVKLQESGGGLVQSKGSLKLSCASGFTFKTYAMN
(connector peptide)
MVVFVMYYYIAGMPMAQVKLQESGGGLVQSKGSLKLSCASGFTFKTYAMN
MHV 5-mer linker
WVRQAPGEGGLEWVARIRTSNKYATSYADSVKDRFTISRSDDQSMLYLHMNNLKT
MKFLNVALVFVMYYYIAGMPMAQVKLQESGGGLVQSKGSLKLSCASGFTFKTYAMN
VH
MKFLNVALVFVMYYYIAGMPMAQVKLQESGGGLVQSKGSLKLSCASGFTFKTYAMN
VL
MKFLNVALVFVMYYYIAGMPMAQVKLQESGGGLVQSKGSLKLSCASGFTFKTYAMN
CQDQQSPRTGAGKLEIKRGTLEIKRPRFEGKPIPNPLLGDSTRTG
Figure 3. Synthesis, expression, and purification of recombinant anti-chitinase 1C3 single-chain antibody for functional assays. A, Schematic representation and translated sequence of synthetic 1C3 single-chain antibody gene as constructed in the insect cell expression vector pMIB/V5-HisB. VH and VL indicate heavy and light variable segments, respectively, derived from the Plasmodium falciparum chitinase PfCHT1–neutralizing, monoclonal antibody (MAb)–producing hybridoma 1C3. B, Western immunoblot using horseradish peroxidase–labeled anti–V5 epitope MAb demonstrating secreted recombinant 1C3 ScFv. Lane 1 contains the culture supernatant, and lane 2 contains lysate of the cell pellet. C, Coomassie blue staining of affinity-purified recombinant 1C3 ScFv. Lane 3 shows the total protein in supernatant, and lane 4 shows the imidazole-eluted recombinant 1C3 ScFv protein.

amplified from template cDNA using the following primers: 1C3-ScFv5'-CATGCATGCTATGGCCCAGGTGAAACTGCAG and 1C3-ScFv3'-TCCCCGGGCGGCGTTTTATTTCTCA-ACTTTGTCC. The reaction conditions were 30 cycles at 94°C for 30 s, 50°C for 30 s, and 68°C for 30 s. The VH and VL products were gel-purified, quantified, and assembled into a single gene containing a DNA linker fragment by use of a 2-step polymerase chain reaction (PCR) protocol. The first reaction was 7 cycles at 94°C for 1 min, 63°C for 4 min, and 72°C for 1 min; the second reaction was 30 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The product of the full-length ScFv gene synthesis reaction was purified with a blunt-ended microspin column (10 mmol/L ATP, 10 mmol/L dNTP, 10 U of T4 DNA polymerase, and 10 U of T4 kinase), was cloned into pUC18, and was electroporated into Escherichia coli DH10B cells. The identity of recombinant ScFv gene–containing colonies was confirmed by SfiI and NorI restriction analysis and sequencing. For expression, the 1C3 ScFv gene was digested with the restriction enzymes SplI and SacII and was cloned into the insect cell expression vector pMIB/V5-HisB (Invitrogen), which carries the N-terminal honeybee melittin secretion signal for directing extracellular secretion of recombinant fusion proteins and a C-terminal peptide containing the V5 epitope and the His6 tag for detection and purification of the recombinant proteins, respectively.

High Five insect cells were transfected with 2 μg of pMIB/V5-HisB-1C3-ScFv using 9 μL of CellFECTIN Reagent (Invitrogen). Transfected cells were seeded into a 6-well tissue culture plate in 2 mL of Ultimate Insect serum-free medium and were incubated at 27°C. The insect cells and the medium were
harvested at a density of $3 \times 10^6$ cells/mL. Cell lysates and culture medium were tested for 1C3 ScFv expression by Western immunoblot analysis, and anti-V5 epitope MAb (Invitrogen) was used for detection. Clonal cell lines were selected with Blasticidin S at a concentration of 50 $\mu$g/mL and were maintained at a concentration of 10 $\mu$g/mL. The pMIB/V$_5$-HisB vector constitutively expresses protein under the control of the transactivating OpOE2 promoter of Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus [11]. Recombinant 1C3 ScFv was purified with nickel-affinity chromatography and was analyzed by 4%–20% SDS-PAGE. Its identity was confirmed by Edman degradation amino-terminal sequencing.

**Immunofluorescence microscopy.** Parasites were fixed on glass slides with 100% acetone at −20°C and were rehydrated by immersion of slides in 2 changes of 1× PBS at room temperature for 5 min/change. For membrane permeabilization and blocking of nonspecific binding, fixed cells were incubated in PBS/3% bovine serum albumin/3% Triton X-100 at room temperature for 1 h. The preparations were then incubated with recombinant 1C3 ScFv (5 $\mu$g/mL) at room temperature for 1 h and then were incubated with fluorescein isothiocyanate (FITC)-labeled anti–V5 epitope MAb (1:200 dilution; Invitrogen) in PBS/3% bovine serum albumin/3% Triton X-100. Slides were mounted with Gelvatol (Molecular Probes). Microscopic examination was performed with a DeltaVision deconvolution immunofluorescence microscope system (Applied Precision).

**Binding of recombinant 1C3 ScFv to P. falciparum chitinase PfCHT1.** Soluble, enzymatically active, $E. coli$–produced, recombinant PfCHT1 was expressed as described elsewhere [3]. Ninety-six–well Immunolon 2 microplates (Dynex) were coated with 4 $\mu$g/mL purified recombinant PfCHT1 in 100 mmol/L sodium carbonate buffer overnight at 4°C, were washed with PBS/0.05% Tween 20, were blocked with PBS/0.05% Tween 20/5% nonfat dry milk, and then were incubated with recombinant 1C3 ScFv, 1C3, and negative control isotype antibody for 3 h at 37°C. Binding was detected using horseradish peroxidase–labeled anti–V5 epitope MAb and 3′,3′,5′,5′-tetramethylbenzidine (Sigma catalog no. T8865), and the optical density at 450 nm was measured.

**Neutralization of recombinant PgCHT2 and PfCHT1 enzymatic activity by recombinant 1C3 ScFv.** Soluble, enzymatically active, $E. coli$–produced, recombinant PgCHT2 was expressed in $E. coli$ strain Rosetta-gami 2 by cloning the postsecretory signal peptide open-reading frame of PgCHT2 into the expression vector pET32b (Novagen). Recombinant PgCHT2 was purified with nickel-affinity chromatography, and elution of protein was performed with 300 mmol/L imidazole. Recombinant PgCHT2 or PfCHT1 was added at a concentration of 1 $\mu$g/mL to individual wells of 96-well black plates to yield linearly increasing enzymatic activity over 30 min, as determined by a kinetic microfluorimetric assay. Recombinant 1C3 ScFv, 1C3, and an IgG1 MAb of irrelevant specificity (against dengue virus and used as an isotype negative control) were added to each well (final volume, 200 $\mu$L). The mixture was incubated for 30 min. Chitinase substrate (10 $\mu$L of 125 $\mu$mol/L 4-methylumbelliferyl-$N,N,N''$-triacetylchitotrioside; Sigma) was then added, and the enzyme reaction was monitored by kinetic fluorescence detection (Dynatek Fluorolite 1000; excitation, 365 nm; emission, 450 nm).

**Experimental feeding of mosquitoes to assess malaria transmission–blocking properties of antibodies.** To assess the effect that recombinant 1C3 ScFv has on $P. falciparum$ infectivity in mosquitoes, in vitro–cultivated $P. falciparum$ strain 3D7 gametocytes (a mixture of day 14 and day 17 gametocytes) were used in membrane feeding assays to colonized Anopheles gambiae strain G3 and Anopheles stephensi mosquitoes. Membrane feeding assays of $P. falciparum$ were performed using glass membrane feeders.

To assess the effect that recombinant 1C3 ScFv has on $P. gallinaceum$ infectivity in Aedes aegypti mosquitoes, 40 female $Aedes aegypti$ mosquitoes, aged 5 or 7 days postemergence, were placed in individual cartons, were starved for 24 h, and then were fed on infectious blood combined with the various antibodies for 20 min. The preparation was maintained at 37°C in water-jacketed membrane feeders. After 15 min of feeding,
Figure 5. Binding and chitinase neutralization activity of 1C3 single-chain antibody against PfCHT1 and PgCHT1. A, Binding of recombinant 1C3 ScFv to recombinant Plasmodium falciparum chitinase PfCHT1. A total of 20 μg/mL in a volume of 200 μL/well of isotype control, 1C3, or purified recombinant 1C3 ScFv was incubated in wells coated with rPfCHT1 and was detected with horseradish peroxidase–labeled anti–V5 epitope monoclonal antibody (MAb) using 3,3′,5,5′-tetramethylbenzidine substrate, and the optical density was measured at 450 nm. B, Measurement of chitinase activity in PfCHT1. Varying quantities of recombinant 1C3 ScFv or an isotype-matched irrelevant MAb were added to a fixed quantity of recombinant PfCHT1, and chitinase activity was measured by a kinetic microfluorimetric assay using 4-methylumbelliferyl-N,N′,N′′-b-D-triacetylchitotrioside as substrate. Enzyme activity is reported as arbitrary relative fluorescence units per minute. C, Measurement of chitinase activity in PgCHT1. Varying quantities of recombinant 1C3 ScFv were added to a fixed quantity of recombinant PgCHT2, and chitinase activity was measured as described above. Error bars show the mean ± SE. Binding and enzyme neutralization experiments were repeated 3 times with equivalent results.

RESULTS

Identification and analysis of the P. gallinaceum and P. reichenowi orthologues of the P. falciparum chitinase PfCHT1. Indirect evidence has suggested the existence of a second chitinase gene in the avian malaria parasite P. gallinaceum [3, 8, 10]. By use of the amino-acid sequence of PICHT1 in a BLAST search of the newly completed P. gallinaceum 3× coverage genome database, a sequence of a second chitinase gene, PgCHT2, was found. Independent sequencing of a cDNA clone obtained from P. gallinaceum ookinetes confirmed the genomic and predicted amino-acid sequence of PgCHT2 (figure 1). Similarly, the complete genomic sequence of the PICHT1 orthologue, PrCHT1, was found in the P. reichenowi genome database. Analysis of the predicted primary amino-acid sequences of PgCHT2 and PrCHT1 confirmed that these 2 genes were orthologues of PICHT1 (figures 1 and 2). All 3 are short forms of Plasmodium species chitinase, which lack both proenzyme and putative chitin-binding domains found in the long form of the chitinase (figures 1 and 2A), have a shared signal peptide length of 24 aa—rather than of 20 aa, as in all long forms of chitinase—and have a tyrosine rather than a tryptophan adjacent to the perfectly conserved proton-donating glutamic acid in the active site of the enzyme (figure 1). Furthermore, the epitope that was recognized by 1C3 and was previously mapped to recombinant PICHT1 is present in both PgCHT2 and PrCHT1 (figure 1) but is absent in the long forms of the chitinase in other Plasmodium species [8]. An unrooted phylogenetic tree of the conserved catalytic region further supports the clustering of PgCHT2, PICHT1, and PrCHT1 at a distance from the other chitinases (figure 2B). This analysis also showed that PgCHT1, the other chitinase of P. gallinaceum, is more closely related to the primate malaria parasite chitinases PvCHT1 and PkCHT1 than to the 3 rodent malaria parasite chitinases (figure 2B). The predicted catalytic domains of PICHT1 and PgCHT2 have 72% amino-acid identity, which is greater identity than the predicted catalytic domains of PICHT1 and PgCHT2, whose amino-acid sequences were only 37% identical.

Construction and expression of the single-chain antibody IC3 gene recognizing P. falciparum PICHT1. The cDNAs encoding the VH and VL segments of the P. falciparum chitinase PICHT1–neutralizing 1C3 were separately amplified by reverse-transcription PCR from mRNA obtained from cloned 1C3 hybridoma cells. The VH and VL cDNAs were synthesized into a single gene using a DNA linker encoding (Gly 4 Ser)3. The synthetic gene was cloned into the insect cell expression vector pMIB/V5-HisB, which is fused at its 5′ end to the honey bee unengorged mosquitoes were removed. The remaining mosquitoes were maintained with 10% sucrose at 26°C and 70%–80% relative humidity until dissection of midguts 7 days after the feeding. Data were analyzed by the Mann-Whitney U test.
Table 1. Effect of the anti-chitinase single-chain monoclonal antibody 1C3 on infectivity of Plasmodium falciparum gametocytes in Anopheles gambiae and A. stephensi mosquitoes.

<table>
<thead>
<tr>
<th>Mosquito vector, experiment, antibody</th>
<th>Geometric mean oocyst count (range)</th>
<th>No. of infected mosquitoes/no. dissected (%)</th>
<th>P</th>
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<td>A. gambiae</td>
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<tr>
<td>1</td>
<td>200 μg/mL isotype control</td>
<td>1.05 (0–9)</td>
<td>15/33 (46)</td>
</tr>
<tr>
<td></td>
<td>200 μg/mL 1C3</td>
<td>0.08 (0–3)</td>
<td>3/35 (9)</td>
</tr>
<tr>
<td></td>
<td>200 μg/mL recombinant 1C3 ScFv</td>
<td>0.06 (0–2)</td>
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</tr>
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<td>14/34 (41)</td>
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<tr>
<td></td>
<td>200 μg/mL 1C3</td>
<td>0.13 (0–3)</td>
<td>4/29 (14)</td>
</tr>
<tr>
<td></td>
<td>200 μg/mL recombinant 1C3 ScFv</td>
<td>0.16 (0–4)</td>
<td>4/31 (13)</td>
</tr>
<tr>
<td>A. stephensi</td>
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<td></td>
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<td></td>
<td>200 μg/mL 1C3</td>
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<td>2/33 (6)</td>
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<td></td>
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<td>16/30 (53)</td>
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<td></td>
<td>200 μg/mL 1C3</td>
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<td>5/33 (15)</td>
</tr>
<tr>
<td></td>
<td>200 μg/mL recombinant 1C3 ScFv</td>
<td>0.15 (0–3)</td>
<td>5/32 (16)</td>
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NOTE. Statistical significance was determined by use of the Mann-Whitney U test. P ≤ .01 was considered to be statistically significant. A total of 200 μL of P. falciparum-infected, gametocyte-containing blood was placed into each membrane feeder, with a final concentration of antibody as noted. Unengorged mosquitoes were removed immediately after membrane feed. Mosquitoes that had fed were dissected 7 days after feeding.

mellitin secretory signal sequence and at its 3′ end to the V5 epitope followed by a His tag (figure 3A). This construct was verified by sequencing.

The pMIB/V5-HisB-1C3-ScFv plasmid was used to transform High Five insect cells and stable transformants that were selected by Blasticidin S drug pressure and cloned. A stably transfected clonal line constitutively secreted recombinant 1C3 ScFv into the culture medium (figure 3B). Affinity-purified recombinant 1C3 ScFv, which was purified to apparent homogeneity as assessed by Coomassie blue staining of SDS-PAGE–separated protein (figure 3C), was used in additional experiments to assess binding to recombinant PfCHT1 and the effect on P. gallinaceum and P. falciparum infectivity in mosquitoes.

Indirect immunofluorescence detection of recombinant 1C3 ScFv binding to PgCHT2 in P. gallinaceum ookinetes. Deconvolution indirect immunofluorescence assay (IFA) microscopy was performed to determine whether recombinant 1C3 ScFv was bound to PgCHT2 expressed by mature P. gallinaceum ookinetes. Binding of recombinant 1C3 ScFv was detected with an FITC-labeled anti-V5 epitope MAb. As was observed with the parental MAb 1C3, recombinant 1C3 ScFv was localized to an apical structure in the ookinete (figure 4B). In the negative control experiments, the FITC-labeled anti-V5 epitope MAb did not significantly bind to ookinetes in the absence of recombinant 1C3 ScFv (figure 4A), which indicates that the indirect IFA was specific for PgCHT2, the target of the single-chain antibody.

Recombinant 1C3 ScFv binding to and neutralizing of recombinant P. falciparum chitinase PfCHT1. The binding of affinity-purified recombinant 1C3 ScFv was determined by ELISA with E. coli–produced recombinant PfCHT1, the antigen against which the parental MAb 1C3 was raised. Recombinant 1C3 ScFv was produced as a fusion protein with the V5 epitope, to allow for specific detection. Recombinant 1C3 ScFv bound specifically to recombinant PfCHT1, as detected with an anti-V5 epitope MAb (figure 5A); binding of an isotype control MAb and the parental MAb 1C3 were indistinguishable from background levels of binding.

The ability of recombinant 1C3 ScFv to neutralize recombinant PfCHT1 and PgCHT2 chitinase activity was determined using a kinetic microfluorimetric assay that measured the release of free 4-methylumbelliferone from 4-methylumbelliferyl-N,N′,N′′-β-d-triacetylchitotrioside [3]. The parental MAb 1C3 has been shown elsewhere to inhibit PfCHT1 enzymatic activity [8]. Recombinant 1C3 ScFv neutralized the enzymatic activity of both PfCHT1 (figure 5B) and PgCHT2 (figure 5C) in a dose-dependent manner.

Determination of malaria transmission–blocking activity of recombinant 1C3 ScFv. The ability of recombinant 1C3 ScFv to inhibit sporogonic development of P. falciparum and P. gal-
DISCUSSION

We have demonstrated that the chitinase of the human malaria parasite *P. falciparum* and the chimpanzee malaria parasite *P. reichenowi* are the orthologues of a newly identified chitinase of the avian malaria parasite *P. gallinaceum*. *P. gallinaceum* is the only *Plasmodium* species that has been demonstrated, to date, to have 2 chitinase genes. An insect cell–produced recombinant single-chain antibody recognized, bound to, and neutralized the activity of both the *P. falciparum* chitinase PfCHT1 and the *P. gallinaceum* chitinase PgCHT2 and reduced the infectivity of both *P. falciparum* and *P. gallinaceum* in mosquitoes. These data—taken together with previous demonstrations that 1C3, an anti-PfCHT1 MAb, has transmission-reducing activity for both *P. falciparum* [9] and *P. gallinaceum* [8]—provide further support for the relevance of the *P. gallinaceum–Aedes aegypti* model for understanding the biochemical mechanisms of how chitinase allows the *P. falciparum* ookinete to invade the mosquito midgut.

The data presented here validate the first effector molecule against *P. falciparum* with the potential for creating a malaria transmission–refractory transgenic *Anopheles* species mosquito. Other anti-*Plasmodium* effector molecules, including single-chain antibodies, the SM1 peptide, and a honeybee phospholipase, have been validated in animal models of malaria transmission. These molecules have been demonstrated to reduce the infectivity of *P. berghei* in *Anopheles stephensi* mosquitoes [12–14], and a virus-expressed single-chain antibody reduced *P. gallinaceum* sporozoite infectivity in *Aedes aegypti* mosquito salivary glands [15]. To our knowledge, this is the first validation of an effector molecule for creating a *P. falciparum*–refractory transgenic mosquito. An important consideration in future experiments with recombinant 1C3 ScFv or any other *P. falciparum* transmission–blocking molecule is whether such interventions would have to be completely effective to justify

<table>
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<tr>
<th>Experiment, antibody</th>
<th>Geometric mean oocyst count (range)</th>
<th>No. of infected mosquitoes/no. dissected (%)</th>
<th>( P )</th>
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<td>PBS</td>
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<td>32/32 (100)</td>
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<td>33/33 (100)</td>
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<td>34/40 (86)</td>
<td>.03</td>
</tr>
<tr>
<td>90 ( \mu )g/mL 1C3</td>
<td>18 (0–103)</td>
<td>34/40 (79)</td>
<td>.007</td>
</tr>
<tr>
<td>50 ( \mu )g/mL recombinant 1C3 ScFv</td>
<td>11 (0–60)</td>
<td>35/40 (88)</td>
<td>.0001</td>
</tr>
<tr>
<td>90 ( \mu )g/mL recombinant 1C3 ScFv</td>
<td>4.5 (0–71)</td>
<td>25/37 (68)</td>
<td>.0001</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>6.7 (0–110)</td>
<td>28/40 (70)</td>
<td></td>
</tr>
<tr>
<td>90 ( \mu )g/mL isotype control</td>
<td>6.0 (0–72)</td>
<td>30/40 (75)</td>
<td>.4</td>
</tr>
<tr>
<td>50 ( \mu )g/mL 1C3</td>
<td>5.4 (0–108)</td>
<td>25/34 (74)</td>
<td>.3</td>
</tr>
<tr>
<td>90 ( \mu )g/mL 1C3</td>
<td>1.6 (0–87)</td>
<td>15/37 (41)</td>
<td>.002</td>
</tr>
<tr>
<td>50 ( \mu )g/mL recombinant 1C3 ScFv</td>
<td>1.3 (0–55)</td>
<td>13/40 (33)</td>
<td>.0004</td>
</tr>
<tr>
<td>90 ( \mu )g/mL recombinant 1C3 ScFv</td>
<td>0.8 (0–35)</td>
<td>10/40 (25)</td>
<td>.00006</td>
</tr>
</tbody>
</table>

NOTE. Statistical significance was determined by use of the Mann-Whitney \( U \) test. \( P \leq .01 \) was considered to be statistically significant. A total of 200 \( \mu \)L of *P. gallinaceum*–infected, gametocyte-containing blood was placed into each membrane feeder, with the final concentration of antibody as noted. Unengorged mosquitoes were removed immediately after membrane feed. Mosquitoes that had fed were dissected 7 days after feeding.
studies of _P. falciparum_ transmission in _Anopheles_ species mosquitoes, particularly at the level of the peritrophic matrix, and have future relevance to the development of malaria transmission–blocking strategies.

Previous phylogenetic analyses of _Plasmodium_ species using ribosomal RNA gene sequences [18–20] have strongly suggested that _P. falciparum_ and the closely related _P. reichenowi_ share an ancestor with avian malaria parasites. However, these analyses did not functionally relate the evolutionary relationship of the phylogenetic lineages of the _Plasmodium_ genus to either a vertebrate or a mosquito host. The present analysis of the phylogenetic relationships between chitinase genes of the different clades of the _Plasmodium_ genus provides specific insight into parasite-vector interactions. The ookinete-secreted chitinase is only known to function in parasite penetration of the peritrophic matrix within the midgut of mosquitoes. Because the mosquito is the definitive host of _Plasmodium_ species, our observations suggest that an important speciation event occurred at the parasite-mosquito interface. The most parsimonious explanation for the relationship between the chitinases of _P. falciparum_ and _P. reichenowi_, on the one hand, and the chitinases of _P. vivax_, _P. knowlesi_, and the rodent malaria parasites, on the other hand, is that an avian-type malaria parasite, or an ancestor that gave rise to avian-type malaria parasites, was the ancestor of both. During the process of adaptation to new mosquito vectors, the long chitinase (in _P. falciparum_ and _P. reichenowi_) or the short chitinase (in the other parasites) was lost, which resulted in only 1 chitinase gene in either lineage. The functional significance of the different chitinase forms vis à vis penetration of the peritrophic matrices of different mosquito species remains to be understood at the biochemical level, although data have been published that suggest that varying tropisms in _Plasmodium_ species for different species and genera of mosquitoes is not likely due simply to the parasite’s ability to penetrate any specific type of peritrophic matrix [21].

The demonstration of the transmission-blocking properties of the single-chain antibody 1C3 for both _P. falciparum_ and _P. gallinaceum_ has 2 major implications. First, the 1C3 ScFv gene may be an additional useful effector molecule for creating a malaria transmission–refractory transgenic mosquito. The malaria transmission–blocking activity of either recombinant 1C3 ScFv or the parental MAb 1C3 was not absolute when the protein was added to a blood meal. However, if recombinant 1C3 ScFv were to be continuously secreted into the midgut after ingestion of an infectious blood meal and concentrated at the blood meal/peritrophic matrix interface immediately adjacent to the epithelial lining, its malaria transmission–blocking effect would likely be longer lasting and more complete than when the antibody (either in the form of a MAb or a recombinant ScFv) is added once to an experimental infectious blood meal, because under those circumstances it is subject to proteolytic degradation. Second, that the 1C3 MAb and ScFv cross-react with both the PfCHT1 and the PgCHT2 chitinase and have transmission-blocking effects supports the functional relevance of the _P. gallinaceum_ model for transmission-blocking studies of _P. falciparum_, particularly at the level of how ookinetes interact with the peritrophic matrix. It is unlikely that recombinant 1C3 ScFv interferes with the activity of PfCHT1, the other _P. gallinaceum_ chitinase, because we have shown that the parental MAb 1C3 does not react with this protein, as determined by Western immunoblot, and PgCHT1 lacks the 1C3–recognized epitope [8], so that the effect of 1C3 is specific for PfCHT1/PgCHT2. It is unlikely that recombinant 1C3 ScFv would have binding specificity, particularly a new specificity for PgCHT1, that the parental MAb does not have.

The demonstration that recombinant 1C3 ScFv has chitinase-binding and chitinase-neutralizing activity provides insight into potential mechanisms of its malaria transmission–blocking effects. Given the monovalency and the lack of an intact Fc component of single-chain antibodies, neither surface molecule cross-linking nor complement-mediated lysis is likely to be responsible for the malaria transmission–blocking properties of recombinant 1C3 ScFv. Therefore, the most likely mechanism by which recombinant 1C3 ScFv blocks parasite transmission is neutralization of secreted [3] or cell surface–associated [8] chitinase enzymatic activity. Given the orthogonal relationship between PgCHT2 and PfCHT1, these mechanistic insights from the _P. gallinaceum_ model system are likely applicable to _P. falciparum_.

The experimental results described here provide additional justification that the ookinate-expressed chitinase of _Plasmodium_ species is a malaria transmission–blocking target and validate a gene encoding a _P. falciparum_ chitinase–neutralizing single-chain antibody as an effector gene that could be used for creating a _P. falciparum–refractory transgenic mosquito_. Targeting sequential molecular processes of parasite infection of the mosquito midgut, before oocyst formation when parasite numbers are exponentially amplified, may be synergistic in the development of a malaria parasite–resistant transgenic mosquito. Therefore, the strategy of targeting a relatively late event in midgut invasion—ookinete penetration of the peritrophic matrix mediated by chitinase—will be complementary to the targeting of the zygote/ookinete surface molecule Pf625 and ookinete mechanisms of binding to and penetrating the mosquito midgut epithelial cell [22].
Acknowledgments

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References

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