VIRUS-EXPRESSED, RECOMBINANT SINGLE-CHAIN ANTIBODY BLOCKS SPOROZOITE INFECTION OF SALIVARY GLANDS IN PLASMODIUM GALLINACEUM-INFECTED AEDES AEGYPTI

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Abstract. Transgenic mosquitoes resistant to malaria parasites are being developed to test the hypothesis that they may be used to control disease transmission. We have developed an effector portion of an antiparasite gene that can be used to test malaria resistance in transgenic mosquitoes. Mouse monoclonal antibodies that recognize the circumsporozoite protein of Plasmodium gallinaceum can block sporozoite invasion of Aedes aegypti salivary glands. An anti-circumsporozoite monoclonal antibody, N2H6D5, whose corresponding heavy- and light-chain variable regions were engineered as a single-chain antibody construct, binds to P. gallinaceum sporozoites and prevents infection of Ae. aegypti salivary glands when expressed from a Sindbis virus. Mean intensities of sporozoite infections of salivary glands in mosquitoes expressing N2scFv were reduced as much as 99.9% when compared to controls.

INTRODUCTION

Malaria is a mosquito-borne disease caused by protozoan parasites in the genus Plasmodium. It exacts a tremendous toll on humans causing more than two million deaths annually.

Previous efforts to control malaria have focused on prophylactic and therapeutic drugs to prevent infection and disease in humans and mosquito control to prevent transmission of pathogens. These approaches have been effective in many areas of the world, but have failed to eradicate the disease. Furthermore, some regions of Africa benefited only modestly from malaria control practices even at the peak of their success. Recently, while efforts to develop malaria vaccines have intensified, the increases in resistance of parasites to drugs and mosquitoes to insecticides have qualified malaria as an emerging disease in areas where it once was controlled.

The threat of malaria has promoted research to develop new control strategies exploiting vector genetics and physiology. One proposed approach is to control transmission by modifying the competence of a mosquito to support the development of parasites. The hypothesis of this approach is that the introduction of an antiparasite gene at a sufficient frequency into a population of vector mosquitoes should result in less transmission, and therefore less disease. Efforts to test this hypothesis focus on three research areas: 1) the development of parasite-resistant mosquito strains, 2) discovery of methods for introducing antiparasite genes into populations, and 3) study of target populations so that the behavior and efficacy of introduced genes can be predicted and monitored.

We are working with Aedes aegypti mosquitoes and the avian malaria parasite, Plasmodium gallinaceum, as an experimental model in order to develop and test many of the basic methodologies that will be applied later to the human malaria parasites and their anopheline vectors. We isolated and characterized mosquito genes whose promoters can be used to express an antiparasite coding region. We also developed stable transformation technology that would allow the integration of antiparasite genes into the mosquito germline. Here we report the development of an effective antiparasite gene.

Malaria parasites are ingested when mosquitoes feed on an infected vertebrate host. Gametocytes produced in the host develop into male and female gametes in the mosquito midgut, and following fertilization, develop from a zygote to a motile ookinite that traverses the single-cell layer of the midgut and develops on the basal surface of the epithelial cells. The developing parasite forms an oocyst in which several thousand sporozoites are produced. The sporozoites break free from oocysts, make their way through the hemolymph, the fluid medium of the open circulatory system of the insect, and invade the salivary glands. They then are inoculated into a new vertebrate host during the next blood meal. We have focused on the sporozoites as the targets of an antiparasite gene at the time that they transit the hemolymph from the midgut to the salivary glands.

The molecular target on the sporozoite is the circumsporozoite protein (CSP), the predominant surface antigen of this parasite stage and one of a number of putative parasite ligands that may mediate recognition and invasion of the salivary glands. Mouse monoclonal antibodies (MAbs) reactive to the CSP of P. gallinaceum block sporozoite invasion of salivary glands, as well as prevent the infection of vertebrate host cells. We engineered molecularly a single-chain antibody (scFv), designated N2scFv, that is based on an anti-CSP MAb, N2H6D5. Expression in vivo of N2scFv by a Sindbis virus vector results in a marked reduction of the mean intensity of sporozoite infections of mosquito salivary glands.

MATERIALS AND METHODS

Mosquito rearing and maintenance of the parasite life-cycle. The RED strain of Ae. aegypti is highly-susceptible to P. gallinaceum, and was used in all experiments. Mosquitoes were reared using standard laboratory procedures.

An aliquot of frozen chicken blood infected with P. gallinaceum strain 8A was obtained from B. Christensen (University of Wisconsin, Madison, WI). This sample was used to inoculate and establish initial infections in chickens. All subsequent infections of chickens and mosquitoes were accomplished by having mosquitoes feed on the chickens. The
maintenance and care of experimental animals complies with National Institutes of Health guidelines for the humane use of laboratory animals.

**Construction of single-chain antibody genes.** Mouse hybridoma cells were cultured in RPMI 1640 (GIBCO BRL, Rockville, MD) supplemented with 10% fetal calf serum. To construct the antinatural scFv gene, total RNA was purified from the N2H6D5 hybridoma cell line using Trizol (GIBCO BRL) and mRNA prepared using the Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ). The mRNA was quantified and an aliquot was used in a commercial gene amplification protocol optimized for scFv gene production (Amersham Pharmacia Biotech). Briefly, oligonucleotide primers based on conserved nucleotide sequences in the variable regions of the heavy-chain (V_H) and light-chain (V_L) immunoglobulin genes were used to synthesize the first strands of corresponding cDNA molecules using reverse transcriptase and dNTPs. A second primer complementary to a nucleotide sequence specific to the V_H or V_L cDNA was added to the appropriate reaction, and gene amplification was used to produce a large amount of the specific fragment. An oligonucleotide linker region (L) with sequence at its 5'-end complementary to the V_L fragment and at its 3'-end complementary to the V_H fragment, and containing nucleotides encoding three repeats of Gly,Ser, was used to assemble a 5'-V_L-L-V_H-3' fragment, which then was amplified quantitatively. The final product, N2scFv, was cloned into the pCANTAB-5E (Pharmacia Biotech) plasmid for further analysis. This plasmid adds an epitope tag (E-tag) to the recombinant protein that permits rapid detection and identification and contains the 22 amino acids of the putative secretion product, which added an E-tag to the recombinant protein.

To synthesize the expression construct, this fragment was cloned into the Bac Dual Expression vector, GIBCO BRL, modified to contain an enhanced green fluorescent protein (EGFP) marker gene, pFBG3 (Hice R and Atkinson P, unpublished data). Again, the identity of all constructs was verified by primary sequence determination of the relevant DNA. Baculovirus-expressed recombinant protein was produced according to the instructions of the manufacturer.

**Protein detection and indirect immunofluorescence analysis.** Proteins analyzed in immunoblot assays were solubilized in Laemmli buffer, resolved in SDS-PAGE gels, and then stained either with Coomassie Blue R or transferred to nitrocellulose membranes. The membranes containing the transferred proteins were incubated in 1:100 dilutions of commercial primary antibodies that recognize the E-tag (Amersham Pharmacia Biotech) or goat antimouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Secondary antibodies conjugated with peroxidase and the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech) were used to visualize the resolved proteins.

*Plasmodium gallinaceum* sporozoites analyzed by indirect immunofluorescence were recovered from the hemolymph of Sindbis virus-infected adult mosquitoes 10 days after a blood meal on parasite-infected chickens. Parasites in the hemolymph were released by gently separating the abdomen from the thorax in a drop (~75 μL) of *Aedes* saline (20 mM HEPES, 150 mM NaCl, 3.3 mM KCl, 2.2 mM MgCl₂, 2.2 mM CaCl₂, 1.8 mM NaHCO₃, pH 7.0). The diluted hemolymph was transferred to a microcentrifuge tube using a mechanical pipette. Hemolymph preparations from a number of mosquitoes were pooled, and sporozoites were pelleted in a microcentrifuge at low speed. The parasites were resuspended in *Aedes* saline, pelleted again by centrifugation, resuspended in 50 μL of *Aedes* saline and an aliquot quantified using a hemocytometer. One thousand sporozoites each were placed in wells of multispot antigen slides and kept at −20°C until use. The slides were fixed with a 4% formaldehyde solution in 10 mM phosphate buffer, pH 7.3, containing 0.13 M NaCl (PBS) for 30 min at room temperature (RT). After careful washing of the wells with drops of PBS containing 0.075% NP40, the slides were incubated for 20 min at RT with 1% bovine serum albumin (PBS/BSA). Slides were incubated for one hour at RT with the E-tag antibody diluted 1:100 in PBS/NP40. The slides were washed with PBS/BSA and incubated for 30 min at RT with a 1:50 dilution of fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG. After the final wash, the slides were dried, mounted in Fluormount (Southern Biotechnology Associates, Birmingham, AL), and examined using UV microscopy.

**Sindbis virus cloning and expression of scFv in mosquitoes.** A 72 base pair (bp) region (nt +57 to +128) of the *Maltaise-like I* (Mal I) gene encoding the translational initiation codon and the 22 amino acids of the putative secretory signal peptide was added to the 5'-end of the N2scFv cDNA to facilitate secretion into the hemolymph of the virally-expressed protein. The addition of these sequences was performed in a two-step gene amplification reaction using the scFv cDNA cloned in the pCANTAB-5E plasmid as a template and the following oligonucleotide primers: Primer 1) 5'-ACTAACCACCGGTTGGACTGGTGGGAAGCCC-3'; Primer 2) 5'-GACTATGCGGCACGCGG-3'; Primer 3) 5'-AGGTGCAACTGCAGGAGTC-3' and Primer 4) 5'-V_L-V_H-L-V_L-3'. The following specificity nucleotide sequences in the variable regions of the heavy-chain and light-chain genes were fixed with a 4% formaldehyde solution in 10 mM phosphate buffer, pH 7.3, containing 0.13 M NaCl (PBS) for 30 min at room temperature (RT). After careful washing of the wells with drops of PBS containing 0.075% NP40, the slides were incubated for 20 min at RT with 1% bovine serum albumin (PBS/BSA). Slides were incubated for one hour at RT with the E-tag antibody diluted 1:100 in PBS/NP40. The slides were washed with PBS/BSA and incubated for 30 min at RT with a 1:50 dilution of fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG. After the final wash, the slides were dried, mounted in Fluormount (Southern Biotechnology Associates, Birmingham, AL), and examined using UV microscopy.

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ANTIBODY BLOCKING OF SPOROZOITE INFECTION

and prepared for gel electrophoresis. Whole proteins in homogenates were resolved in gels and visualized by staining with Coomassie Blue R (left). An identical gel was prepared and blotted to a filter (right). The filter was incubated in the presence of the commercial antiEtag antibody (Etag) and exposed to film. A signal corresponding to a single predominant polypeptide is evident in the mosquito extracts derived from N2scFv and 4G2scFv-expressing mosquitoes and is not present in the controls (NoSin). The locations of molecular weight markers are shown to the right of the figure.

**FIGURE 1.** Expression and binding of N2scFv to sporozoites in mosquitoes. A) Schematic representations of expression fragments cloned into the pTE/3/H11032 plasmid. The thin horizontal lines represent the Sindbis viral vector with the 5’- and 3’-end orientation indicated. The large arrowhead segments represent the Sindbis virus promoter (Sindbis promoter). The regions of DNA encoding the secretory signal peptide (Mal I signal peptide), enhanced Green Fluorescent Protein (EGFP), single-chain antibody coding region (N2scFv or 4G2scFv), and epitope tag (Etag) are indicated in their proper 5’- to 3’-end orientation. B) Detection of Sindbis-expressed scFvs in homogenates of virally-infected adult mosquitoes. Mosquitoes infected with a Sindbis virus expression construct were homogenized and prepared for gel electrophoresis. Whole proteins in homogenates were resolved in gels and visualized by staining with Coomassie Blue R (left). An identical gel was prepared and blotted to a filter (right). The filter was incubated in the presence of the commercial antiEtag antibody (Etag) and exposed to film. A signal corresponding to a single predominant polypeptide is evident in the mosquito extracts derived from N2scFv and 4G2scFv-expressing mosquitoes and is not present in the controls (NoSin). The locations of molecular weight markers are shown to the right of the figure. C) Immunodetection of N2scFv binding to sporozoites in vivo. Spermatogonia were recovered from the hemolymph of Plasmodium gallinaceum-infected mosquitoes that had been injected with SinN2scFv or a control virus, SinEGFP. Spermatogonia were incubated with the E-tag antibody and fluoroscein-conjugated secondary antibody, and examined using Phase contrast or Fluorescence microscopy. Only spermatogonia exposed to N2scFv react with the appropriate antibody.

**RESULTS**

**Sindbis virus-mediated expression of N2scFv in mosquitoes.** Immunoblot analyses demonstrated that recombinant N2scFv bound to CSP (de Lara Capurro M and James...
AA, unpublished data). We determined next whether N2scFv cloned into a Sindbis virus construct was expressed in mosquitoes and bound sporozoites in situ. SinN2scFv was injected into adult female mosquitoes (Figure 1A). Control mosquitoes were untreated or injected with either SinEGFP or Sin4G2scFv. Whole mosquitoes were homogenized 10 days after viral infection, and the homogenates resolved by SDS-PAGE and analyzed on immunoblots (Figure 1B). Proteins of the predicted molecular weights, ~30 kiloDaltons (kDa) for N2scFv and ~29 kDa for 4G2scFv, were detected in only those mosquitoes infected with SinN2scFv or Sin4G2scFv, respectively. These results demonstrated that the scFvs were expressed in the mosquito.

Additional experiments were conducted to determine if the expressed N2scFv bound sporozoites (Figure 1C). Following infection by SinN2scFv or control viruses, and a blood meal from a parasite-infected chicken, parasites were recovered from the hemolymph and reacted with the E-tag antibody. Only those parasites from mosquitoes expressing N2scFv reacted with the E-tag antibody. These results demonstrated that N2scFv was produced as a result of viral expression in the mosquitoes and that the expressed recombinant antibody reacted with sporozoites in vivo.

### Table 1

<table>
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<tr>
<th>Experiment</th>
<th>Group</th>
<th>Prevalence of midgut infection (no.)</th>
<th>Estimated range or mean intensity of oocysts in midgut</th>
<th>Prevalence of salivary gland infection (no.)</th>
<th>Mean intensity of sporozoites in salivary glands</th>
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<td>100 (3/3)</td>
<td>15–50</td>
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<td>84 (16/19)</td>
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<td>44.3 ± 32.3</td>
<td>100 (5/5)</td>
<td>57.1 ± 36.1</td>
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* Percentage of the number of infected dissected tissues divided by the total number of dissected tissues.
† Mean intensity is the average number of parasites per tissue determined for only those tissues with an infection.
§ % reduction is determined by comparing the mean intensities in SinN2scFv-infected groups with the SinEGFP groups in each experiment (Expts. 1–6), or the SinN2scFv-infected groups with the Sin4G2scFv groups (Expts. 7–9).
\* n.d. = not determined.

### N2scFv blocks sporozoite infection of salivary glands.

A series of nine experiments were performed to determine if Sindbis virus-expressed N2scFv could block sporozoite infection of salivary glands (Table 1). The number of oocysts were estimated or counted to provide an initial indication of the intensity of infection in a specific experiment. Mosquitoes in Experiments 1–4 were estimated to have high oocyst intensities of infection, greater than 15 oocysts/midgut, and the intensity of infection in a specific experiment. Mosquitoes in Experiments 5 and 6 had lower oocyst intensities, and again the controls were either SinEGFP-infected or NoSin mosquitoes. The mosquitoes in Experiments 7–9 had variable oocyst intensities of infection, and the controls used were NoSin and Sin4G2scFv-infected mosquitoes.

Injections of virus in Experiments 1–6 had no effect on oocyst prevalence or mean intensity of infection. However, Sin4G2scFv-infected mosquitoes (Experiments 7–9) showed a consistent reduction in oocyst mean intensities when com-
pared to NoSin controls. It appeared that virus infection did not affect the prevalence of sporozoite infections in salivary glands in any of the experiments. It is important to emphasize that prevalence was determined by including salivary gland pairs that were infected by as few as one sporozoite. In all experiments, control viruses reduced by about one-half the sporozoite intensity of infections in salivary glands. In contrast, mean intensities of sporozoites in salivary glands from SinN2scFv-infected mosquitoes were reduced by at least 96.8% in all experiments, and in one, Experiment 7, sporozoites were reduced by 99.9% when compared to virus-infected controls.

A histogram that represents the percentage of mosquitoes in each treatment group from all experiments that had a particular level of infection is shown in Figure 2. These data show that 53% (54/102) of the pairs of salivary glands recovered from SinN2scFv-infected mosquitoes had 10 or fewer sporozoites while less than 14% (31/228) of the mosquitoes in the combined control groups had similar values. Furthermore, a total of 84% (86/102) of the salivary gland pairs from the SinN2scFv group had 100 or fewer parasites, and this value contrasts remarkably with those obtained for the corresponding NoSin controls. In addition, virus infection had an effect on the mean intensities of salivary gland infections among controls. In contrast, those mosquitoes with no or a low titer virus infection should result in high numbers of parasites in their glands. The distribution of the data plotted in Figure 2 is consistent with this interpretation. However, it is important to emphasize that the overall effect of N2scFv was to reduce substantially the mean intensities of sporozoite infections of salivary glands.

Mean intensities of sporozoite infections in virus-expressing control mosquitoes (SinEGFP or Sin4G2scFv) were about half of those observed in the corresponding NoSin controls. In addition, virus infection had an effect on the mean intensity of oocysts in Sin4G2scFv-infected mosquitoes. We interpret these results to be due to the cytopathological effects of the virus expression. Damage to cells in the midguts and salivary glands may account for these differences.

Experiments 1–6 used SinEGFP-infected mosquitoes as negative controls. The structure of this construct lacks the Mal 1 secretory leader peptide and E-tag sequences. Therefore, for Experiments 7–9, we used Sin4G2scFv as a control. No consistent differences in percent reduction in mean intensities of salivary gland infections among controls were observed indicating that the leader peptide and E-tag sequences played no role in the blocking properties of N2scFv.

The data presented here cannot be used to determine the level of sporozoite blocking that is required to interrupt parasite transmission. Estimates vary as to the minimum sporozoite inoculum required during a natural infection cycle to establish an infection in a host. For some Plasmodium species, 10 or fewer sporozoites may be sufficient. However, a reliable correlation has not been established between the mean intensity of infection in salivary glands and the number of sporozoites inoculated during feeding. Although we have achieved reduction of the mean intensities to 100 or fewer parasites per gland for 84% of the N2scFv-expressing mosquitoes, 16% of the mosquitoes have greater than this amount and these are sufficient to transmit parasites to the vertebrate host (de Lara Capurro M and James AA, unpub-
lished data). We conclude that although the Sindbis expression system allows testing the potential of an antiparasite effector molecule, because of its variability in infection, it is not adequate to test transmission blocking. Stable, transgenic lines expressing N2scFv will be needed to do the latter experiments. Individual transformed lines should express N2scFv at a constant level (depending on the promoter used to drive the expression of the construct and the site of genome integration), and parasite intensity of infection can be measured as a function of the level of N2scFv present.

We do not know the mode of action of N2scFv that is responsible for the reduction of the mean intensity of sporozoite infections in the salivary glands. Although domain I of the CSP has been shown to be important in binding of Plasmodium falciparum to Anopheles stephensi salivary glands, the repeat region of P. gallinaceum CSP to which the parental N2H6D5 MAb binds, has yet to be shown to be a true ligand domain. Binding of N2scFv to CSP may interfere with other receptor-ligand interactions and prevent the parasite from contacting the salivary gland. Alternatively, N2scFv may immobilize or kill sporozoites in some manner that remains to be determined. However, it is evident from the data presented here and other published blocking data that the repeat region can be targeted to interrupt parasite transmission.

A number of proteins have been tested as potential effector molecules for engineered antiparasite genes. Insect defensins, magainins and cercepin-like molecules, and scorpion toxins have all been shown to kill malaria parasites. An scFv that targets the Plasmodium berghei ookinete protein, Pbs21, blocks ookyst development in the mosquito if the recombinant antibody is injected into a mouse host and is ingested by the mosquito along with the parasite. These and similar molecules may be used in conjunction with scFvs like N2scFv to introduce into mosquitoes a synthetic, multifunctional antiparasite gene that may prevent resistance mechanisms from developing rapidly in the parasites. Of course, application of these developments to P. falciparum and Plasmodium vivax is the ultimate goal, and we anticipate that these results along with those of other investigators will encourage research in these important human pathogens and their vectors.

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