Monoclonal antibodies AC-43 and AC-29 disrupt *Plasmodium vivax* development in the Indian malaria vector *Anopheles culicifacies* (Diptera: culicidae)

MANOJ CHUGH[1*], B R GULATI[2] and S K GAKHAR[3]

[1]*National Institute of Pharmaceutical Education and Research, Ahmedabad 380 054, India*

[2]*National Research Centre on Equines, Sirsa Road, Hisar 125 001, India*

[3]*Advanced Centre for Biotechnology, M D University, Rohtak 124 001, India*

**Corresponding author (Fax, +91-79-27450449; Email, manoj.chugh2@gmail.com)*

A repertoire of monoclonal antibodies (mAbs) was generated against the midgut proteins of *Anopheles culicifacies* mosquitoes. The mAbs AC-43 and AC-29 significantly inhibited *Plasmodium vivax* development inside the mosquito midgut. The number of oocysts that developed was reduced by 78.6% when mosquitoes ingested a combination of these two mAbs along with the blood meal. AC-43 mAb binds to the epitope common in 97, 80 and 43 kDa polypeptides from the midgut protein extract, as indicated by western blot analysis. Similarly, the mAb AC-29 recognized 52, 44, 40 and 29 kDa polypeptides. These female midgut-specific polypeptides are shared between *An. culicifacies* and *An. stephensi,* two major vectors of malaria in India. Deglycosylation assays revealed that *O*-linked carbohydrates are the major components in epitopes corresponding to AC-43 and AC-29. Gold particle labelling revealed that both these mAbs preferentially bind to glycoproteins at the apical microvilli and the microvillus-associated network present inside transverse sections of the gut epithelium. These regions are particularly known to have receptors for ookinetes, which enable them to cross this epithelial barrier and provide them with certain necessary chemicals or components for further development into oocysts. Therefore, these glycoproteins appear to be potential candidates for a vector-directed transmission-blocking vaccine (TBV).

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1. Introduction

Mosquitoes are vectors of many fatal or debilitating diseases, none more challenging than malaria, which claims over a million lives annually (Snow *et al.* 2005). In this era of environmental changes, resurgent diseases, and drug and insecticide resistance, there is an urgent need to explore alternative or novel control strategies. Most of the malaria vaccines currently under development target forms that are found in humans, such as pre-erythrocytic and erythrocytic stages of the parasite (Carter 2001). Development of malaria vaccines that block the transmission of parasites by mosquito vectors remains one such pragmatic approach that can complement existing control methods. Vaccines are also being developed from the parasite antigens expressed during their sexual stages in the mosquito midgut (Duffy and Kaslow 1997; Hisaeda *et al.* 2000). However, malaria transmission can also be blocked by targeting mosquito components that are required for the successful development of the parasite inside the vector (Lal *et al.* 1994; Shahabuddin *et al.* 1996; Lal *et al.* 2001; Dinglasan *et al.* 2003; Lavazec *et al.* 2007).

Keywords. *An. culicifacies*; midgut; mosquito; *Plasmodium*; transmission blocking

Abbreviations used: ELISA, enzyme-linked immunosorbent assay; mAB, monoclonal antibody; MMV, midgut microvillar; PBS, phosphate buffered saline; PMSF, phenyl methyl sulphonyl fluoride; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBV, transmission-blocking vaccine

The midgut is one of the most attractive sites for developing transmission-blocking vaccines (TBV). The midgut epithelium is the first and most important barrier that the parasite encounters in the mosquito. Failure to invade this tissue may confer refractoriness to the *Plasmodium* parasite. The mosquito midgut also represents one of the most challenging environments for the survival and development of *Plasmodium* (Shahabuddin and Costero 2001).

Previous studies have demonstrated that polyclonal antibodies (Abs) raised against the midgut not only blocked parasite (*P. vivax*) development but concomitantly disrupted the reproductive physiology of *An. stephensi* mosquitoes (Suneja *et al.* 2003). A dose-dependent blocking effect of mAb MG96 to midgut microvillar (MMV) glycans of *Aedes aegypti* on *P. yoelli* development in the midgut of *An. stephensi* has been observed (Dinglasan *et al.* 2003). However, discoveries emanating from the use of rodent parasites may not be applicable directly to *P. falciparum* or any other human parasite (Dinglasan *et al.* 2007).

No such study appears to have been carried out in the Indian malaria vector *An. culicifacies*. *An. culicifacies* exists as a complex of five sibling species provisionally designated as A, B (Green and Miles 1980), C (Subbarao *et al.* 1983), D (Vasantha *et al.* 1991) and E (Kar *et al.* 1999). These sibling species are reported to have various biological differences such as their distribution, response to insecticides (Raghvendra *et al.* 1992), host preferences (Kar *et al.* 1999) and vectorial capacity (Subbarao *et al.* 1988). Despite numerous studies, a global view of the ecological and bionomical characteristics of individual sibling species has not been achieved because of the different identification methods used by various researchers. Various studies have unequivocally incriminated *An. culicifacies* A, C and D to be the vectors of *P. vivax* and *P. falciparum* malaria (responsible for 70% of cases in India) and species B to be a poor vector, if at all (Subbarao *et al.* 1988; Barik *et al.* 2009).

In the present investigation, repertoires of mosquito midgut-specific mouse mAbs were screened for their effect on *P. vivax* development in *An. culicifacies* midgut. A combination of mAbs AC-43 and AC-29 significantly disrupted *Plasmodium* development. In addition, epitopes specific to both mAbs were partially characterized. The antigens corresponding to both these mAbs were found to be microvillus-associated midgut glycoproteins.

2. Materials and methods

2.1 *Mosquito rearing*

A cyclic colony of *An. culicifacies* species A (Dhera strain) was maintained in an insectarium at 28± 2°C, 70–80% relative humidity, and fitted with a simulated dawn and dusk machine to maintain a photoperiod of 14 h light and 10 h dark. Adult mosquitoes were held in 30 cc cloth cages and fed on 1% glucose solution and water-soaked resins. Females were allowed to feed on rabbit blood for ovarian development. On the third day post blood-feeding, females were allowed to lay eggs on filter paper that lined water-filled plastic bowls. Larvae were reared in enamel trays and fed on yeast extract and dog biscuits in the ratio of 2:3. After initial development, the pupae were transferred to fresh cloth cages for emergence to adult mosquitoes.

2.2 *Antigen preparation and immunization*

Midguts from glucose-fed *An. culicifacies* species A were dissected out in ice-cold phosphate buffered saline (PBS) containing 2 mM phenyl methyl sulphonyl fluoride (PMSF) at 4°C as described earlier (Suneja *et al.* 2003). All precautions were taken to minimize the activity of proteases. Proteins from the whole body of instar-IV larvae and early stage pupae were also extracted.

2.3 *Generation of mAbs*

A group of six 8–10-week-old female BALB/c mice was immunized with 100 μg of midgut protein extracts with Freund complete adjuvant. After three weeks of primary immunization, mice were again immunized with a similar dose of protein extract along with Freund incomplete adjuvant. Immunizations continued until the enzyme-linked immunosorbent assay (ELISA) titre in serum reached between 64 000 and 128 000. Three days prior to fusion of splenocytes with immortal (SP 2.0) cells, the mice were inoculated intraperitoneally with midgut protein extract.

Hybridoma cells were generated by the polyethylene glycol method with selection in hypoxanthine–aminopterin–thymidine (HAT) medium. The resulting hybridomas were screened for midgut-specific antibodies by indirect antibody capture ELISA (Suneja *et al.* 2003). The isotype of generated mAbs was determined with a mouse monoclonal iso-typing kit (Sigma) according to the manufacturer's instructions.

2.4 *Immunoblotting*

Lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Blots were probed with AC-43 or AC-29 Abs from ascitic fluids at 1: 500 dilution. Bound Abs were detected with an alkaline phosphatase-conjugated goat anti-mouse IgG Ab (Sigma), followed by a chemiluminescent reaction with NBT-BCIP substrate (Sigma). Protein loading and transfer efficiency were monitored by Coomassie blue and Ponceau S staining, respectively (Suneja *et al.* 2003).

2.5 *Immunoelectron microscopy*

Localization of the midgut protein specific to the mAbs was examined by immunoelectron microscopy (Dinglasan *et al.* 2003). Briefly, unfed midguts from 8–10-day-old *An. stephensi* mosquitoes were fixed in PBS containing 1% paraformaldehyde and 0.1% glutaraldehyde for 24 h at room temperature. Ultrathin sections of guts, embedded in LR-White resin, were mounted on nickel grids and immunostained with mAbs raised against midgut proteins of *An. culicifacies*. Antibody binding was visualized by using a 10 nm colloidal gold-conjugated goat anti-mouse IgG. The grids were counterstained with uranyl acetate and examined with a Morgagni-268D transmission electron microscope.

2.6 *Characterization of the epitope recognized by AC-43 and AC-29*

Chemical deglycosylation was carried out as already described (Dinglasan *et al.* 2003). The midgut proteins were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. The membrane was divided into four identical sections and incubated in 50 mM sodium acetate wash buffer (pH 4.5). Sections were used either as control or as experimental/treatment groups. One experimental strip was incubated with 20 mM sodium periodate in 50 mM sodium acetate buffer (pH 4.5) at room temperature for 1 h in the dark. The other experimental strip was incubated in ice-cold 1 mM sodium periodate at 0°C for 1 h in the dark. Controls strips were kept in wash buffer under the same conditions without the addition of sodium periodate (Sigma). The strips were rinsed with three exchanges of wash buffer and then incubated with 50 mM sodium borohydride (in PBS) at room temperature for 30 min. The membranes were washed three times with PBS, followed by the standard detection of antigens by mAbs as described for immunoblot analysis.

For enzymatic deglycosylation, midgut lysates were boiled at 95°C for 10 min and then treated either with 0.5 mU of peptide *N*-glycosidase A (EC 3.5.1.52) or with a mixture of 1 mU of neuraminidase (EC 3.2.1.18) and 1 mU of *O*-glycosidase (EC 3.2.1.97/3.2.1.110) (Sigma). The reactions proceeded overnight in deglycosylation buffer (10 mM Tris, 0.5% SDS, 2% 2-mercaptoethanol, 5% NP-40) at 37°C. Deglycosylated proteins were run on SDS-PAGE and immunoblotted as described above.

2.7 *Parasite invasion-blocking assay*

Membrane feeding was used to examine the effect of anti-midgut mAbs on the development of the parasite in *An. culicifacies*. In each experiment, venous blood (5–10 ml) drawn from human hosts infected with the gametocytes of *P. vivax* was collected in a pre-warmed tube coated with heparin. Infected blood was centrifuged at 37°C for 5 min at 5000 rpm and the plasma discarded. The parasitized erythrocytes obtained were mixed with ascitic fluid from the BALB/c mice containing 100 μg of IgGs as described earlier (Suneja *et al.* 2003). Two groups of 200 mosquitoes (5-day-old) each were membrane fed either on anti-midgut mAbs or isotype-matched non-specific mAbs. Unfed or partially fed females were removed; only fully engorged females were maintained at the insectarium until dissection. After 6 days, the midguts were dissected out to count the number of oocysts. Percentage transmission blocking was determined by the method described elsewhere (Ponnundrai *et al.* 1987; Abraham and Jacobs-Lorena 2004).

All experiments were conducted in triplicate. The significance of the differences in oocyst numbers between mosquitoes in each experiment, and the number of oocyst-infected guts between the experimental and control groups were analysed by the Mann–Whitney *U* test, one-tailed, by using the GRAPH-PAD Prism software.

3. Results and discussion

3.1 *AC-43 and AC-29 mAbs recognized distinct midgut proteins*

The antibodies in the ascitic fluid raised from the BALB/c mice injected with the hybridoma cell line AC-43 and AC-29 were capable of binding to the midgut proteins of *An. culicifacies* as well as *An. stephensi* as seen on western blot analysis. AC-43 mAb was associated with the epitope common in three polypeptides (97, 80 and 43 kDa) present in the midgut of female *An. culicifacies* and *An. stephensi* mosquitoes (figure 1A). Similarly, AC-29 was specific to the epitope present in four polypeptides (52, 44, 40 and 29 kDa) of female midguts from both the vectors (figure 1B). Smaller polypeptides recognized by mAbs AC-43 and AC-29 were not necessarily processed/degradation products. The presence of similar peptides was confirmed when separation of the proteins was carried out in non-reducing conditions on native PAGE but the molecular weight of the peptides observed on SDS-PAGE cannot be compared with the peptides observed on native PAGE because of entirely different conditions and non-availability of molecular weight markers for native PAGE. The detection of multiple protein bands by mAbs is not uncommon and has been demonstrated in various other studies (Woodward *et al.* 1985; Hanisch *et al.* 2001; Wang *et al.* 2001; Takeuchi *et al.* 2002; Dinglasan *et al.* 2003). Addition of carbohydrate moieties to newly synthesized glycoproteins after post-translational

modification is known to affect the molecular weight of proteins, as analysed by SDS-PAGE. To understand the recognition specificity of AC-43 and AC-29, protein lysates from *An. culicifacies* carcasses minus the midguts were also immunoblotted (figure 1). The results demonstrated that these mAbs bind specifically to the female mosquito midgut. An isotype-matched mAb control did not reveal any non-specific recognition of midgut antigens.

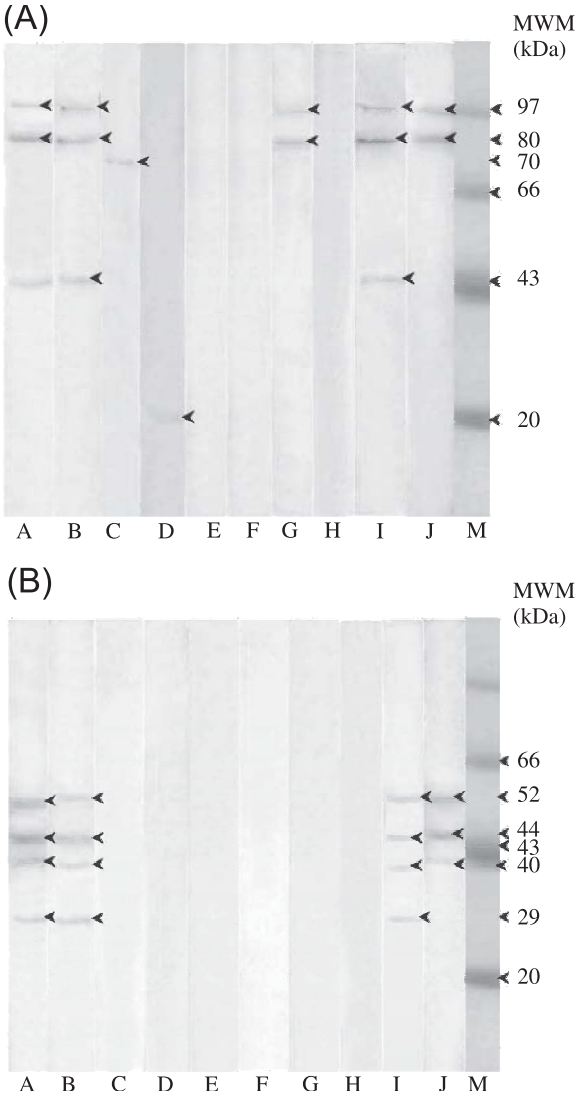

Figure 1. Immunological reactivity of **(A)** AC-43 and **(B)** AC-29 to the protein lysates from (A) midgut of *An. culicifacies*, (B) midgut of *An. stephensi*, (C) instar-IV larvae, (D) Early pupae, (E) carcasses minus midgut of *An. culicifacies*, (F) male, (G) midgut extracts following chemical deglycosylation with 1 mM sodium periodate, (H) midgut extracts following chemical deglycosylation with 20 mM sodium periodate, (I) midgut extracts following treatment with 0.5 mU of PNGase A, (J) midgut extracts following treatment with 1mU of neuraminidase plus 1 mU of *O*-glycosidase and (m) Molecular weight marker.

The AC-43 mAb was also capable of recognizing a 70 kDa peptide in the mixed-sex population of instar-IV larvae and a 20 kDa peptide in early pupae of *An. culicifacies* (figure 1A). However, the AC-29 mAb did not recognize any peptide either in larvae or in pupae (figure 1B).

One of the most important antibody characteristics is their isotype. The isotype of each mAb generated was determined by an isotyping kit based on indirect antibody capture ELISA. MAbs AC-43 and AC-29 (with transmission-blocking activity) were isotypized as IgG2a and IgG2b, respectively.

3.2 *Epitopes recognized by both mAbs AC-43 and AC-29*

Partial characterization by enzymatic and/or chemical deglycosylation assays suggested the presence of carbohydrate moieties as one of the major components of these epitopes.

Sodium periodate (1 mM at 0°C) preferentially cleaves terminal neuraminic acid from both *N*- and *O*-linked glycans, whereas treatment with 20 mM sodium periodate cleaves off all oligosaccharides from the protein backbone. The AC-43 recognition profile was unaffected following treatment with 1 mM sodium periodate; however, treatment with 20 mM sodium periodate completely eliminated recognition by AC-43. However, the AC-29 recognition profile was completely altered by even 1 mM sodium periodate (figure 1). Chemical deglycosylation assays revealed that AC-43/AC-29 mAbs recognize epitopes that have carbohydrates as a major component.

Treatment of mosquito midgut lysates with PNGase A, an N-linked oligosaccharide-Asn-specific exoglycosidase, did not alter the recognition pattern of mAbs AC-43 and AC-29 (figure 1). However, 43 kDa and 29 kDa proteins were not recognized by their respective Abs after initial treatment of midgut lysates with a non-specific neuraminidase to remove terminal neuraminic acid, followed by treatment with *O*-glycosidase, an *O*-linked Gal (1,3) GalNAc-*α*-Ser/Thr-specific endoglycosidase. These results suggest that the epitope is complex and may be composed primarily of *O*-linked carbohydrates.

In contrast to *N*-glycosidases, *O*-glycosidases specifically cleave the Gal (1,3) GalNAc disaccharide attached to serine and threonine residues (Kramerov *et al.* 1997; Dinglasan *et al.* 2003). Although various different glycoproteins are reported to be present in the midgut of *Anopheles*, only a limited repertoire of carbohydrate moieties are added onto the proteins as they cycle through the Golgi apparatus (Seppo and Tiemeyer 2000). Several studies have shown that midgut proteins in particular are heavily *O*-glycosylated (Kramerov *et al.* 1997; Seppo and Tiemeyer 2000; Wilkins and Billingsley 2001).

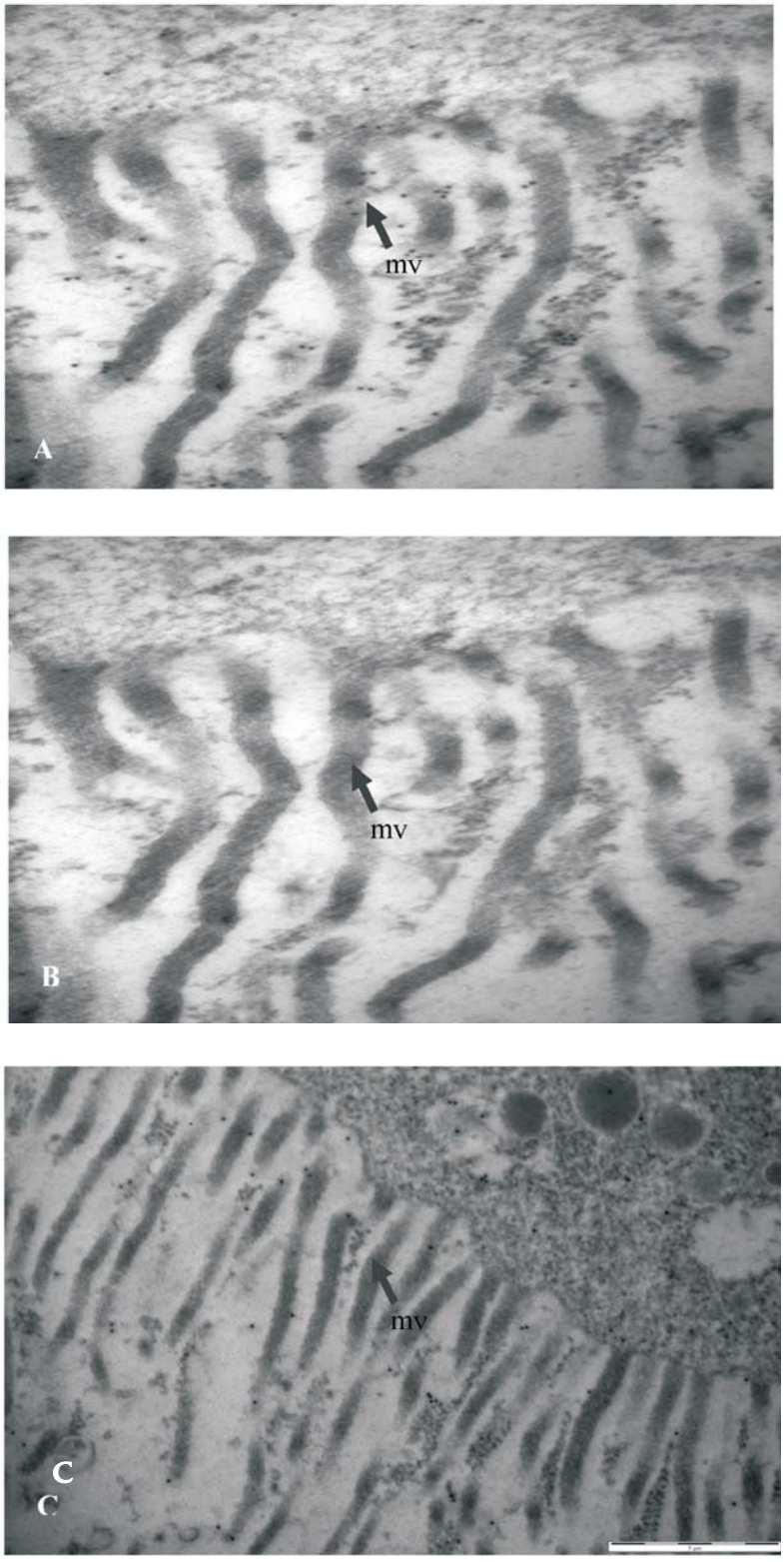

Figure 2. Immunoelectron microscopy of unfed *An. culicifacies* midguts. Transverse section of the midgut showing the association of **(A)** AC-43 and **(B)** AC-29 with the extracellular microvillar region (arrowhead) on the apical end of the midgut epithelial cell. **(C)** Transverse section of the midgut, showing the microvillar region after staining with isotype-matched control mAbs. MV, microvilli. Magnification ×15 000.

The other proteins, i.e. ~97 and ~80 kDa, recognized by AC-43: ~52, ~44 and ~40 kDa recognized by AC-29, remained unaffected even after treatment with *O*-glycosidase (figure 1). This could presumably be ascribed to inefficient enzyme digestion, as the rate of enzymatic hydrolysis is much lower for larger glycoproteins. Another possibility would be that the epitope is composed of both carbohydrate and protein, as reported earlier (Figueroa *et al.* 1990; Kramerov *et al.* 1996; Dinglasan *et al.* 2005). Glycosylation contributes up to 40% of the molecular weight of mature glycoproteins in invertebrates (Figueroa *et al.* 1990; Hanisch 2001) and as much as 50–80% in mammals (Figueroa *et al.* 1990; Kramerov *et al.* 1996; Takeuchi *et al.* 2002). Glycosylation profile has also been reported to influence mAb binding to glycoproteins (Figueroa *et al.* 1990; Takeuchi *et al.* 2002; Dinglasan *et al.* 2003).

3.3 *Localization of antigens*

Immunoelectron microscopy was performed to determine the precise subcellular localization of antigens recognized by AC-43 and AC-29 in the midgut of *An. culicifacies* (figure 2). Gold particle labelling revealed that both these mAbs preferentially bind to glycoproteins at the apical microvilli and microvillus-associated network present inside the transverse sections of the gut epithelium. Immunostaining with an isotype-matched control Ab to a serial cross-section of the midgut showed no background labelling of either extracellular or intracellular components.

Gut epithelial cells are the first to confront invading ookinetes from their apical side. Oligosaccharides present on the gut microvilli have already been implicated as both receptors for microbial attachment and as protective barriers against pathogens in both vertebrates and invertebrates (Figueroa *et al.* 1990; Takeuchi *et al.* 2002). MMV glycoconjugates play a definite role in the establishment of parasitic infections. *Plasmodium* development may be disrupted either at the level of attachment to the microvillus region or at the level of more downstream events following invasion, e.g. interference of cell signalling and/or cell functioning (Gulia *et al.* 2002).

3.4 *Effect of AC-43 and AC-29 on parasite development*

Several doses and combinations of mAbs were tested in parasite transmission-blocking assays. The infection rate of *An. culicifacies* mosquitoes was reduced by about 64% and the mean number of oocysts per infected mosquito was drastically reduced by about 67% ($P<0.01$) when the mosquitoes ingested AC-43 mAb (100 μg/ml) along with *P. vivax.* Similarly, the infection rate of *An. culicifacies* was reduced by about 51% and the mean number of oocysts was reduced by about 42.8% ($P<0.01$) when the mosquitoes ingested AC-29 mAb (100 μg/ml). However, the infection rate was reduced by about 67% and the mean number of oocysts per infected mosquito (*An. culicifacies*) was reduced by about 79% ($P<0.01$) when mosquitoes ingested these two mAbs (100 μg/ml) in combination, i.e. mAbs AC-43 + AC-29 (in a ratio of 1:1). AC-43 mAb blocked ≈ 67% of the total ookinete population and mAb AC-29 blocked 33.5% of the remaining 33% (table 1). The mechanism of action of these antibodies for obstructing the natural course of infection by *Plasmodium* is not well established. The reduction in the number of oocysts developed in the midgut may be due to two reasons; first, the anti-midgut Abs bind to similar target sites which the parasite recognizes to invade the epithelia and therefore competitively inhibit the parasite. Second, these Abs probably adhere and lead to allosteric alteration in the receptor recognition site in the midgut: therefore, ookinetes are unable to cross this barrier (Suneja *et al.* 2003; Dinglasan *et al.* 2007). The reduced infectivity observed in the present study is consistent with that in previous studies where the animals were immunized with whole midguts (Suneja *et al.* 2003; Corfield and Shukla 2001). This observation reveals that the epitopes corresponding to these mAbs (AC-43 and AC-29) probably play the key role in the transmission-blocking activity of whole midguts.

Cross-reactive epitopes that have been preserved during the evolution of arthropods should be the ideal targets to develop a global TBV that works against all the human parasites across different Anopheline species. The presence of some common antigenic polypeptides between different species of mosquitoes as well as various tissues has already been indicated, viz. salivary gland, midgut and haemolymph

Table 1. Effect of monoclonal antibodies (AC-43 and AC-29) raised against the midgut of glucose-fed *An. culicifacies* mosquitoes on malaria parasite (*P. vivax*) development (standard deviation has been mentioned in parentheses)

Number of mosquitoes fed/ group (100 μg/ml)				Infected mosquitoes (%)				Mean number of oocysts/mosquito				Transmission blocking (%)		
Control	AC-43	AC-29	AC-43 + AC-29	Control	AC-43	AC-29	AC-43 + AC-29	Control	AC-43	AC-29	AC-43 + AC-29	AC-43	AC-29	AC-43 + AC-29
90	70	74	80	55.5	20	37.8	18.7	105 (± 5.41)	24.92 (± 2.73)	60 (± 14.25)	23.60 (± 2.54)	67.3*	42.8*	78.6**

* Significant at $P<0.05$; ** Significant at $P<0.01$.

in a given mosquito species (Dinglasan *et al.* 2003; Corfield and Shukla 2001). In comparison with other proteins, the *N*-linked and *O*-linked glycans of arthropods seem to have stalled at an intermediate evolutionary step such that most of the *N*-linked glycans have a paucimannosidic structure and the *O*-linked glycans are limited to the core glycan structures known as Tn and T antigens (GalNAc-Ser/Thr and Galb1-3GalNAc-Ser/Thr, respectively). Cross-reactive antigenicity predominantly due to glycans has been detected in several arthropods. Limited evolutionary diversification and high immunogenicity of these glycans categorize them as key candidates for the development of a vaccine capable of inducing immunity to salivary gland and midgut glycoproteins of all species of haematophagous arthropods. This approach has earlier been suggested as a way to develop vaccines to control arthropod-borne diseases (Willadsen and Billingsley 1996). In the present study, mAbs AC-43 and AC-29 specific to the epitope with *O*-linked carbohydrate as a major component significantly blocked parasite development.

Future investigations of the epitope as well as gene identification should provide valuable insight into the development of the parasite in the midgut or possible mechanisms of ookinete attachment and invasion into mosquito midgut epithelial cells.

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