Mosquitocidal Immune Response in BALB/c Mice Is Enhanced When *Anopheles gambiae* Mucin-1 cDNA Is Co-Administered with Interleukin-12 cDNA

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Abstract

The midgut of the malaria - transmitting mosquito, *Anopheles gambiae,* can be targeted by vaccine-induced host immune factors that kill the mosquito after it ingests immunized host blood. The *An. gambiae* mucin 1 protein (AgMuc1) is expressed on the mosquito midgut where it likely functions in protecting the midgut epithelium from its own secreted digestive enzymes, toxic substances and pathogenic microbes taken in with the blood meal. Immunization of mice with plasmid containing the AgMuc1 gene has been shown to induce mosquitocidal immune responses. In this paper, we co-immunized mice with AgMuc1 cDNA and plasmid containing Murine granulocyte-macrophage stimulating factor (GM-CSF) or Interleukin 12 (IL-12) cytokine cDNA in order to further potentiate the mosquitocidal immune response and better define the nature of this mosquitocidal immunity. While co-immunization with GM-CSF cDNA failed to increase anti-mosquito immunity (Chisq = 3.3 on 1 degree of freedom, $p = 0.068$), a significantly enhanced mosquitocidal effect was observed from mice co-immunized with AgMuc1 and IL-12 cDNA (Chisq = 39.1 on I degree of freedom, $p = 4.06\text{e-}10$). Furthermore, the cumulative survival of the blood fed mosquitoes surviving to day 7 in the AgMuc1/IL-12 co-immunized group highly correlated negatively with the anti-mucin IgG1 antibody subtype levels (Pearson correlation coefficient $r = -0.782$) suggesting that the mosquitocidal immunity induced by AgMuc1 cDNA immunization could be IgG1 antibody subtype mediated.

Keywords: DNA vaccination, immunostimulatory cytokines, mosquitocidal immune response

1. Introduction

Current efforts in the control of malaria are targeted towards the elimination of adult mosquitoes and their larval stages from the environment and the malaria parasites from the human host. These two strategies entail the use of chemicals such as insecticides, larvicides and anti-malaria drugs whose effectiveness has been hampered by the rapid development of resistance by both the parasite and the vector in addition to their harmful effects to the environment and the human body. Alternative mosquito control strategies, including immunological control, have been evaluated (Alger & Cabrera, 1972; Almeida & Billingsley, 1998; Noden, Vaughan, Ibrahim, & Beier, 1995; Ramasamy, Srikrishnaraj, Wijekoone, Jesuthasan, & Ramasamy, 1992). In order to develop anti-mosquito vaccines that kill mosquitoes or that block pathogen transmission to the mosquito, immunological interactions occurring at the molecular level at the interface of the mosquito midgut and the blood meal need to be characterized. It is in the midgut where there is direct contact between most host immune components and potential targets in the epithelium. These targets could be essential to vector physiology; hence any immune

responses to such targets could compromise mosquito survival. The effectiveness of such anti-vector control strategies has already been demonstrated (Riding et al., 1994; Inokuma, Kerlin, Kemp, & Willadsen, 1993) and further shown to prevent parasite development (Lal et al., 2001) and kill the mosquito vector (Lal et al., 2001; Foy et al., 2003).

Crude tick extracts have been used to stimulate acaricidal immune responses in guinea pigs and rabbits (Trager, 1939). Due to the problems of immunodominance, immune masking, and antibody cross- reactivity associated with the use of crude extracts, identifying the specific immune factors responsible for killing is difficult (Foy et al., 2003). The use of highly purified proteins to circumvent these problems proved more effective leading to the production of the first commercially available acaricide vaccine (Riding et al., 1994).

DNA vaccination on the other hand has been shown to elicit both humoral and cellular immune responses *in vivo* upon injection of gene expression cassettes directly into host tissue (Tang, DeVit, & Johnston, 1992; Wang et al., 1993). In the midgut of a blood fed female *An. gambiae*, several potential anti-mosquito antigen targets have been cloned and characterized (Shen, Dimopoulos, Kafatos, & Jacobs-Lorena, 1999) allowing for the possibility of using their cDNA for immunization studies. In an attempt to generate and characterize anti-mosquito immune responses to mosquito antigens, groups of mice were immunized with two individual cDNAs, AgMuc1 and Ag-Per1, and an *A. gambiae* cDNA library (Foy et al., 2003). Significantly increased mortality (Chisq = 95.91, on 1 degree of freedom, p = 0.0001) was observed among mosquitoes that fed on either the AgMuc1- or the cDNA library immunized mice compared to that of controls (Foy et al., 2003).

Immunostimulatory cytokine genes could potentiate the mosquitocidal immune response from the AgMuc1 cDNA immunized mice and allow for further characterization of the immune response responsible for mosquito killing (Kim et al., 1997, 1998). Cytokines influence antigen presentation by MHC class I and class II molecules on antigen presenting cells (APCs) thus modulating the immune response (Lanzavecchia, 1993). IL-12 is a cytokine that plays a critical role in Th1 immune response by mainly inducing the production of IFN-γ by CD4+ T cells (Stern, Magram, & Presky, 1996; Kim et al., 1997). GM-CSF is a hemopoietic growth factor that stimulates and increases the antibody dependent cell mediated cytotoxicity of neutrophils, monocyte/macrophages and eosinophils (Weiss et al., 1998; Valerie, 1998).

We co-immunized mice with AgMuc1 cDNA and plasmids expressing IL-12 or GM-CSF in order to manipulate the immune response so that the biological effects of antibody and cell mediated immune responses on mosquito survival could be determined separately.

2. Materials and Methods

2.1 AgMuc1 Plasmid

The full-length Ag-Aper14 and Ag-Muc1 (GenBank accession no. AJ007394) coding sequences were amplified byPCR and cloned into the pGEX-4T-1 expression vector (Pharmacia Biotech) and full-length Ag-Muc1 expressed as a GST fusion protein in E. coli BL21-CodonPlus (DE3)-RP cells (Stratagene) (Devenport et al., 2005). The *A. gambiae* mucin 1 (AgMuc1) was subcloned from the parental vector into the into ampicillin resistant pcDNA3.1_ plasmid vector (Invitrogen) with Xba1 and Hind III restriction enzymes and electroporated into XL1-Blue *E. coli* and then sequenced prior to immunization to ensure proper orientation and translation (Foy et al., 2003). Plasmid DNA for immunization was harvested from *E.coli* using Qiagen endotoxin-free Giga Prep kits (Valencia, CA) and diluted appropriately in endotoxin-free PBS. The final plasmid preparations were verified to have <0.1 endotoxin units/μg using the chromogenic Limulus Amebocyte Lysate test (Bio Whittaker).

2.2 Murine GM-CSF and IL-12 Plasmids

Murine GM-CSF cDNA in pUMVC1 and Murine IL-12 cDNA in pUMVC3 kanamycin-resistant plasmid vectors (Aldevron, North Dakota) were electroporated into XL1-Blue *E.coli* and the E. coli plated onto LB agar plates with kanamycin. Single colonies were harvested from the LB agar plates with kanamycin and inoculated into LB broth containing kanamycin. Large-scale cultures from the single colonies were prepared in LB broth containing kanamycin. Plasmid DNA was harvested from *E.coli* culture broth using Qiagen endotoxin-free Giga Prep kits (Valencia, CA) and diluted appropriately in endotoxin-free PBS (Foy et al., 2003). The final plasmid preparations were verified to have < 0.1 endotoxin units/μg using the chromogenic *Limulus* Amebocyte Lysate test (Bio Whittaker).

2.3 Plasmid DNA Immunization

Four groups of five female BALB/c mice (6-8 weeks old) per experimental group with an equal number of mice in the control group were immunized as shown in Table 1.

Table 1. Immunization groups

	Group 1	**Group 2**	**Group 3**	**Group 4**
Experimental	200 µg AgMUC1 cDNA	200 µg AgMUC1 cDNA + 100 µg GM-CSF cDNA	200 µg AgMUC1 cDNA + 100 µg IL-12 cDNA	200 µg AgMUC1 cDNA followed by 10 mg recombinant mucin protein boost
Control	200 µg Empty vector cDNA	200 µg Empty vector cDNA+ 100 µg GM-CSF cDNA	200 µg Empty vector cDNA + 100 µg IL-12 cDNA	200 µg Empty vector cDNA followed by 10 mg Ovalbumin protein boost

The mice were obtained from Charles River Laboratories (Bar Harbor, ME) and kept under specified pathogen-free conditions in the Department of Vivarial Sciences at Tulane University Medical School. Pre-immune sera were collected via tail bleed to test for mucin specific antibodies by ELISA. None of the mice tested were found to have anti-mucin antibody activity in their sera. Mice from each group were injected four times, 14 days apart. Before immunization, the AgMuc1 cDNA and the cytokine cDNA were mixed such that 20 µl of the mixture contained 200 µg of AgMuc1 cDNA and 100 µg of the cytokine cDNA. This mixture was given at 20 µl per mouse via the hind footpad. To demonstrate that immunization with plasmid containing cDNA is expressed in mice, both mouse muscle and footpad were immunized with the pcDNA: GFP construct where cryosections of the injection sites showed that muscle fibers expressing GFP were visible 48 h postinjection, and migrating amoeboid-like cells (presumably dendritic cells) expressing GFP were visible in the footpad 24 h postinjection (Foy et al., 2003)

2.4 Mosquito Bioassays

Adult *An. gambiae* G3 strain were reared in the insectary of the Department of Vivarial Sciences at Tulane University Medical School on 10% Karo syrup at 28°C, 75% humidity on a 12-12 hour light - dark cycle. Fourteen days after the last plasmid cDNA boost, approximately 40-60 adult female *An. gambiae* 4-6 days old in clean 500 ml capacity cages were fed on each Ketamin/Xylazine anesthetized mouse. Mosquitoes that did not feed were removed at the end of the feeding session, which lasted 15 to 20 minutes. Day 1-7 post blood feeding, the cages were examined for the number of dead mosquitoes which were recorded and removed. All mosquitoes were given fresh sugar and water pads daily during the 7 day period.

2.5 ELISA for Anti-Mucin Antibody

Immediately after mosquito feeding, serum was harvested from the mice, stored at -80°C until later use. Immulon 96-well micro-ELISA plates (Dynatech, Alexandria, VA) were coated with 2.5 µg recombinant mucin protein in 100 µl carbonate-bicarbonate coating buffer (0.1 M NaHCO$_3$, pH 9.6) per well and incubated at 4°C overnight. The plates were washed twice with a washing buffer, 0.05% Tween 20 (Sigma Chemical Co. St. Louis, MO) in 1X PBS, blocked with a blocking solution (5% BSA-0.05% Tween 20 in 1X PBS) for 1 hour, then washed three times again. An aliquot of 100 µl of serially diluted serum samples were added to each well and incubated at room temperature for 2 hours and washed five times after the reaction. An aliquot of 100 µl of the appropriate horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody subtype (whole molecule; Sigma Chemical Co. St. Louis, MO) diluted 1:6000, was added and incubated at room temperature for 1 hour and then washed five times. Finally, 100 µl of TMB substrate solution (BD Biosciences Pharmigen, San Diego, CA) was added and incubated at room temperature for 30 minutes. The reaction was stopped with the addition of 50 µl of 1M sulphuric acid. Plates were read with a micro plate reader (Labsystem) at 405 nm. The cut-off was calculated using the non-parametric method of two times the mean positive percent of the optical density of the pre-immune sera. The Pearson correlation analysis was used to establish the relationship between mean antibody titres and the daily cumulative survival of the mosquitoes surviving to day 7 for each immunization group. Western blots of various midgut antigens had earlier been probed by using immunized mouse group serum as the primary antibody where it was demonstrated that cDNA immunized mice produced IgG antibodies against protein corresponding to the translated midgut cDNAs with which they were immunized (Foy et al., 2003). That's why only ELISA to determine the strength of the anti-mucin antibodies was performed.

2.6 Determination of Cytokine Levels

Cytokines IL-2, IL-4, IL-5, IFN-γ and TNF-α were determined from supernatants from invitro stimulation of spleen cells from the immunized mice groups. Stimulation was achieved using 15 µg of recombinant mucin

protein in 100 μl of growth medium per well to which 100 μl spleen cell suspension was added. The cytokines were determined using a commercially available quantitative ELISA based test kit using the manufacturers protocol (Pharmigen).

2.7 Data Analysis

The R statistic software version 2.14.2 (2012-02-29) from the R Foundation for Statistical Computing was used for data analysis. Survival analysis was performed using life tables to determine the daily cumulative survival of the blood fed mosquitoes surviving to day 7. Kaplan-Meier statistical analysis and the Log-rank test were used to determine the differences in the daily cumulative survival of the mosquitoes surviving to day 7 when fed on different groups of immunized mice. Bivariate correlations using the Pearson's correlation coefficient (r) and the two-tailed test of significance were performed to establish the relationship between the blood fed mosquito's daily cumulative survival and the specific anti-mucin IgG antibody subtype titres.

3. Results

3.1 Survival Analysis

The *A. gambiae* mosquitoes that fed on immunized mice were monitored for 1 week after their blood meal for changes in mortality. The daily cumulative survival of mosquitoes surviving to day 7 post blood feeding was analyzed by constructing life table survival curves that were compared by the Kaplan Meier log rank analysis in order to obtain the difference and significance of the difference in the survival of mosquitoes fed on different mice groups. No significant differences were observed in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 between groups of mosquitoes fed on individual mice within their immunization groups (AgMUC1 Chisq = 1.5 on 4 degrees of freedom, p = 0.834; Vector Chisq = 3.3 on 4 degrees of freedom, p = 0.511; AgMUC1/GM-CSF Chisq = 3.2 on 4 degrees of freedom, p = 0.531; Vector/GM-CSF Chisq = 7.1 on 4 degrees of freedom, p = 0.13; AgMUC1/IL-12 Chisq = 6.1 on 3 degrees of freedom, p = 0.108; Vector/IL-12 Chisq = 4.7 on 4 degrees of freedom, p = 0.322; AgMUC1/Mucin Chisq = 2.7 on 4 degrees of freedom, p = 0.102 ; AgMUC1/Ovalbumin Chisq = 0.2 on 1 degrees of freedom, p = 0.62). The individual data from the mice in each group were therefore pooled.

Significant differences were observed in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 post blood feeding between groups of mosquitoes fed on the experimental AgMuc1 and the control empty vector immunized groups (Chisq = 29.1 on I degree of freedom, p = 6.72e-08; Figure 1). This was also observed in the experimental AgMuc1/IL-12 and its control empty vector/IL-12 immunized group (Chisq = 39.1 on I degree of freedom, p = 4.06e-10; Figure 2). This means that more mosquitoes are dying when fed on mice immunized with the AgMUC1 cDNA due to the anti-mucin immune responses in these mice.

No significant differences were observed between the daily cumulative survival of the blood fed mosquitoes surviving to day 7 post blood feeding between groups of mosquitoes fed on the experimental AgMuc1/GM-CSF and the control empty vector/GM-CSF immunized group (Chisq = 3.3 on 1 degree of freedom, p = 0.068; Figure 3) and the experimental AgMuc1/mucin protein boost and the control AgMUC1/Ovalbumin boost group (Chisq = 1 on 1 degree of freedom, p = 0.314; Figure 4). The number of mosquitoes dying in the experimental and control groups was high and similar. This was not expected for the experimental AgMuc1/GM-CSF and the control empty vector/GM-CSF immunized group. For the experimental AgMuc1/mucin protein boost and the control AgMUC1/Ovalbumin boost group this was not surprising as there is anti-mucin immune responses in the two groups.
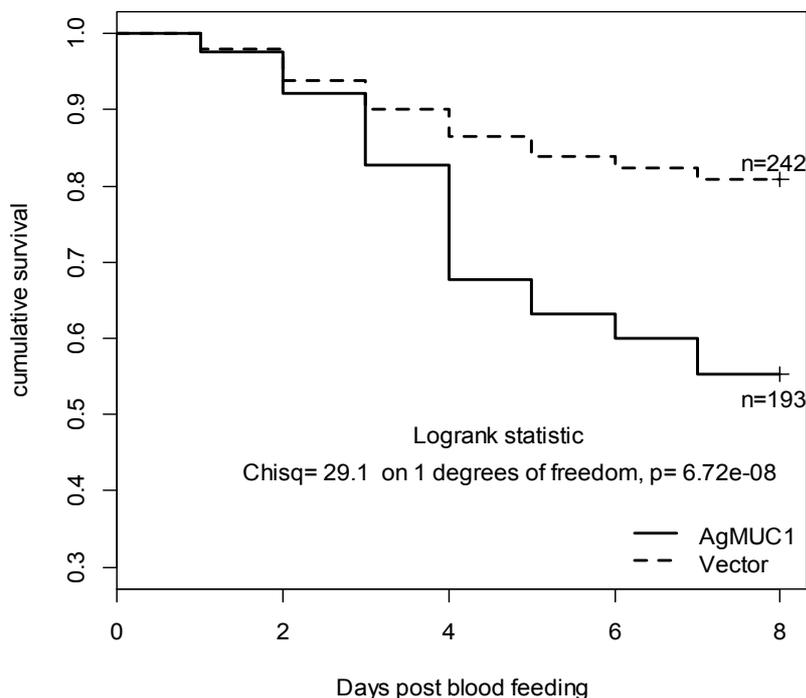
Figure 1. Survival curves of *An. gambiae* mosquitoes fed on mice immunized with AgMUC1 cDNA and empty vector cDNA. Constructed from life tables and compared by Kaplan Meier log rank analysis. Significant differences in survival were observed (Chisq = 29.1 on I degree of freedom, *p* = 6.72e-08*)* between mosquitoes fed on mice immunized with AgMUC1 cDNA and Empty vector cDNA
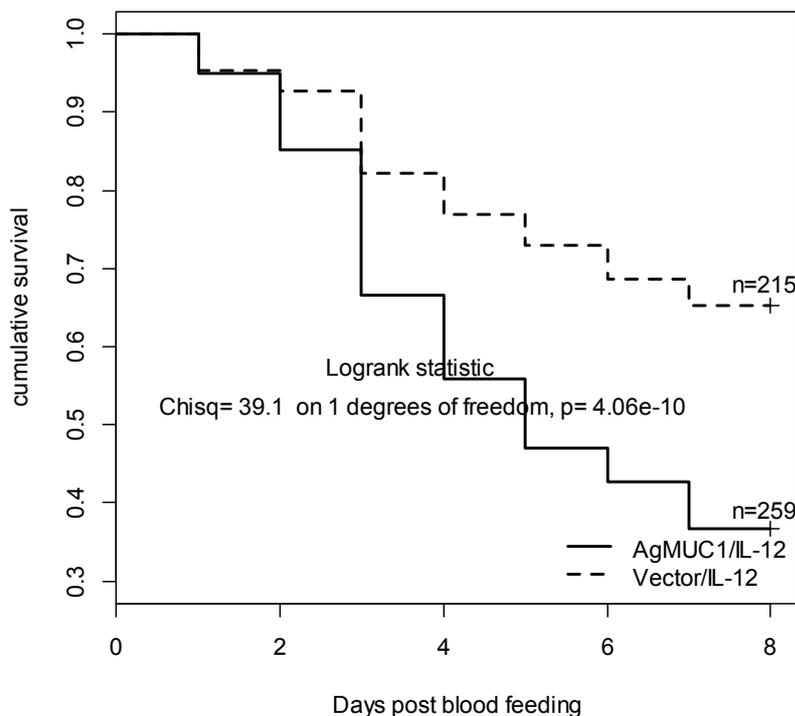
Figure 2. Survival curves of *An. gambiae* mosquitoes fed on mice immunized with AgMUC1 + IL-12 cDNA and Empty vector + IL-12 cDNA. Constructed from life tables and compared by Kaplan Meier log rank analysis. Significant differences in survival were observed (Chisq = 39.1 on I degree of freedom, *p* = 4.06e-10*)* between mosquitoes fed on mice immunized with AgMUC1 cDNA + IL-12 cDNA and Empty vector cDNA + IL-12 cDNA
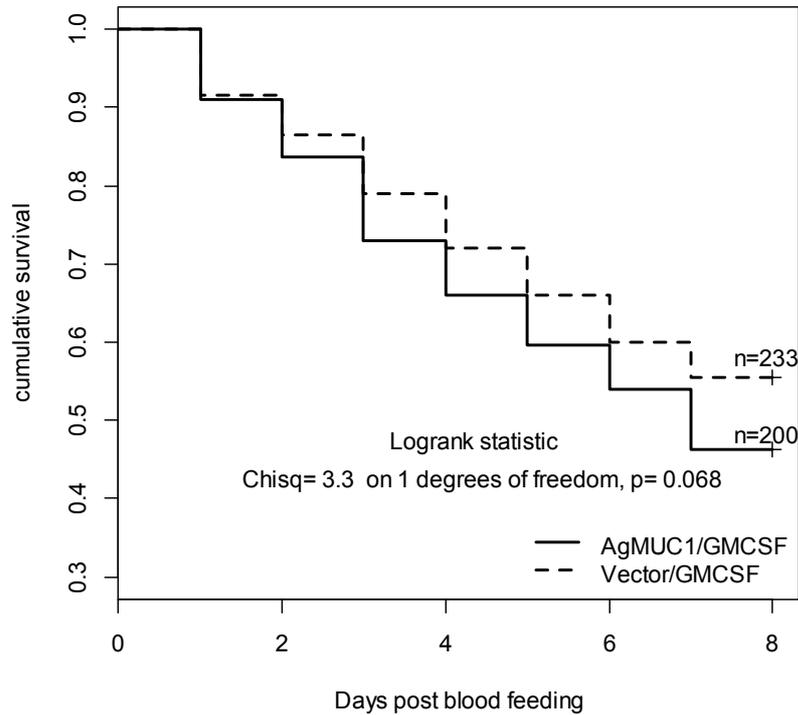
Figure 3. Survival curves of *An. gambiae* mosquitoes fed on mice immunized with AgMUC1 + GM-CSF cDNA and Empty vector+ GM-CSF cDNA. Constructed from life tables and compared by Kaplan Meier log rank analysis. No significant differences in survival were observed (Chisq = 3.3 on 1 degree of freedom, *p* = 0.068*)* between mosquitoes fed on mice immunized with AgMUC1 cDNA+ GM-CSF cDNA and Empty vector cDNA + GM-CSF cDNA
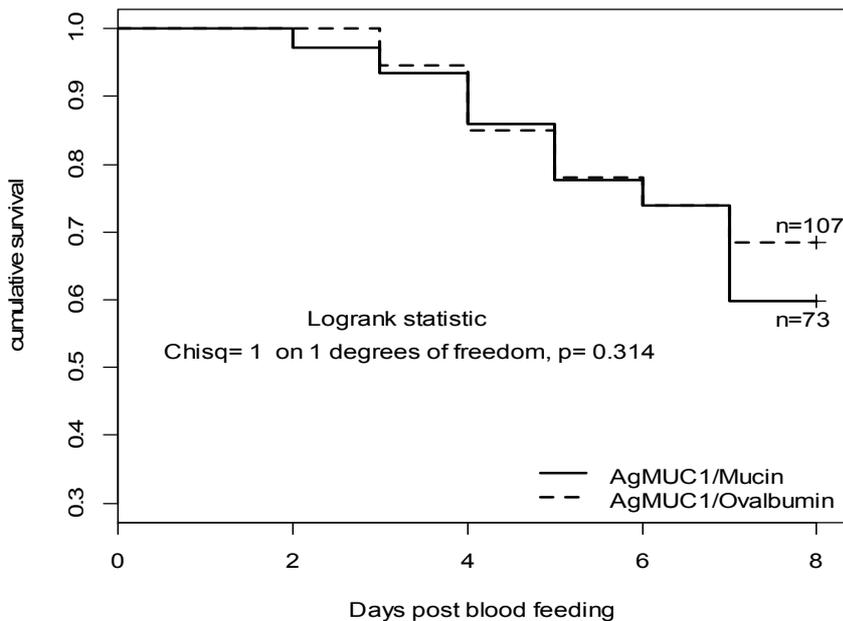
Figure 4. Survival curves of *An. gambiae* mosquitoes fed on mice immunized with AgMUC1 cDNA + a final boost of recombinant mucin protein and AgMUC1 cDNA + OvalAlbumin. Constructed from life tables and compared by Kaplan Meier log rank analysis. No Significant differences in survival were observed (Chisq = 1 on 1 degree of freedom, *p* = 0.314*)* between mosquitoes fed on mice immunized with AgMUC1 cDNA + a final boost of recombinant mucin protein and Empty vector cDNA + OvalAlbumin

3.2 Anti-Mucin Specific Antibody Titres

The mean titre for anti-mucin IgG specific antibodies increased from a titre of 1:3 000 in the AgMuc1 immunized group to 1:6 000 in the AgMuc1/GM-CSF and AgMuc1/IL-12 immunized groups and over 1:100 000 in the AgMuc1/Mucin protein boosted group. In the AgMuc1/Ovalbumin group the titre remained at 1:3 000. Titres in the respective control groups were <1:200 (Table 2). There was a trend whereby there were high negative correlations between the daily cumulative survival of blood fed mosquitoes surviving to day 7 with IgG anti-mucin antibody titres in the AgMuc1/GM-CSF group (r = -0.759, $p > 0.05$) and the AgMuc1/IL-12 group (r = -0.782, $p > 0.05$) (Table 2). All IgG1 antibody titres in the four experimental groups showed high negative correlation values with the mosquito's daily cumulative survival to day 7 (Table 2).

Table 2. Pearson's correlation coefficient analysis (r) between the daily cumulative proportions of *Anopheles gambiae* mosquitoes surviving to day 7 and the IgG antibody and it's subtypes in the various groups of immunized mice

GROUP	IgG Titre (r)	IgG1 Titre (r)	IgG2a Titre (r)	IgG3 Titre (r)
AgMUC1	1:3,000 (0.350)	1:3,000 (-0.582)	1:640 (0.444)	0 (***)
AgMUC1/GM-CSF	1:6,000 (-0.759)	1:3000 (-0.914)	0 (***)	0 (***)
AgMUC1/IL-12	1:6,000 (-0.782)	1:3000 (-0.606)	0 (***)	0 (***)
AgMUC1/Mucin Protein	1:102,400 (0.387)	1:66600 (-0.671)	1:82000 (0.384)	1:1840 (0.144)
AgMUC1/Ovalbumin	1:3,000 (0.617)	1:3000 (-0.758)	0 (***)	0 (***)

3.3 Cytokine Levels

As shown in Table 3 spleen cells from AgMUC1 cDNA immunized mice on *in vitro* stimulation produced higher levels of IL-2 (33 pg/ml compared to 20 pg/ml in the control group), IFN-α (4208 pg/ml compared to 1428 pg/ml in the control group), IL-5 (4 pg/ml compared to 1.4 pg/ml) and TNF-α (689 pg/ml compared to 443 pg/ml in the control group) and no IL-4 (0 pg/ml compared to 2 pg/ml in the control group); AgMUC1/GMCSF cDNA group produced similar levels of IL-2 and IL-4 (35 pg/ml and 0.9 pg/ml respectively in both the experimental and control groups) and lower levels of IFN-y (2020 pg/ml compared to 3680 pg/ml in the control group), IL-5 (4 pg/ml compared to 6 pg/ml in the control group) and TNF-a (469 pg/ml compared to 568 pg/ml in the control group); AgMUC1/IL-12 cDNA group produced slightly higher levels of IFN-γ (4633 pg/ml compared to 4153 pg/ml in the control group), IL-4 (1.1 pg/ml compared to 0.5 pg/ml in the control group) and TNF-α (470 pg/ml compared to 320 pg/ml in the control group and lower levels of IL-2 (31 pg/ml compared to 41 pg/ml in the control group) and IL-5 (1.5 pg/ml compared to 2.7 pg/ml in the control group); AgMUC1 cDNA immunized / Mucin protein boosted mice generally secreted lower levels of IL-2 (24 pg/ml), IFN-y (2652 pg/ml), IL-5 (0 pg/ml), IL-4 (0.5 pg/ml) and TNF-a (356 pg/ml) respectively compared to AgMUC1 cDNA immunized / Ovalbumin protein boosted mice with IL-2 (33 pg/ml), IFN-y (3474 pg/ml), IL-5 (5.7 pg/ml), IL-4 (0.9 pg/ml) and TNF-a (366 pg/ml) respectively.

Table 3. Cytokine profiles and their Mean concentrations from recombinant mucin protein stimulated spleen cells from the immunized mice

Immunisation Group	IL-2 (pg/ml)	IFN-γ (pg/ml)	IL-5 (pg/ml)	IL-4 (pg/ml)	TNF-α (pg/ml)
AgMuc1	33±15	4208±1533	4.0±3.8	0	689±199
Empty vector	20±13	1428±2030	1.4±2.2	2.0±2.0	443±143
AgMuc1+GM-CSF	35±13	2020±947	4.0±2.0	0.9±1.4	469±113
Empty vector + GM-CSF	35±10	3680±1280	6.0±2.0	0.9±1.2	568±107
AgMuc1+IL-12	31±17	4633±604	1.5±3.0	1.1±1.3	470±184
Empty vector + IL-12	41±14	4153±604	2.7±2.8	0.5±1.2	320±75
AgMuc1 + Mucin protein	24±16	2652±1133	0	0.5±1.1	356±50
AgMuc1 + Ovalbumin	33±20	3474±1734	5.7±6.0	0.9±0.8	366±134

4. Discussion

GM-CSF has been reported to improve immune responses through enhancing the production of higher concentration of antibodies following co-administration with plasmid expressing *Plasmodium yoelii* circumsporozoite protein (Weiss et al., 1998). IL-12 has also been reported to modulate the immune response by promoting cell mediated immune mechanisms that protect against pre-erythrocytic stage malaria (Doolan et al., 1999). In this study, these cytokines were administered as plasmids and not as proteins so that they can exert their mechanisms through expression of the cytokine proteins in the host by the transfected cells. This system might not be as vigorous in stimulating the immune response as administering the cytokine protein itself but it was envisaged that the transfected cells would secrete the cytokine over a longer period of time, hence eliminating the need for boosting the immunized mice to continue stimulating the immune response as is the case with the use of recombinant proteins. Furthermore while using plasmids; some cells may be transfected with both the cytokine and the immunization genes thus enhancing the immune response to the immunization gene due to its proximity to the cytokine.

In this study, GM-CSF co immunization was not shown to have enhanced antibody response as expected (Table 2). The antibody titers generated following co immunization with GM-CSF were only two-fold higher than those generated following AgMUC1 cDNA immunization alone (1:3 000 versus 1:6 000). In mice, it has been reported that after intramuscular immunization with plasmids encoding mice GM-CSF, antibody production can vary widely from moderate titers to undetectable antibody levels depending on the encoded antigen (Gardner et al., 1996). Thus the moderate titers obtained in this study after AgMUC1 cDNA co immunization with GM-CSF are not surprising. Other reports have shown that when plasmid encoding mice GM-CSF is mixed with *Plasmodium yoelli* circumsporozoite plasmid and the mixture used to immunize mice, specific antibodies increased 8-fold (Weiss et al., 1998), tremendously improving vaccine efficacy. In this study, following co administration of AgMUCI cDNA and plasmid GM-CSF antibody levels increased only two-fold (Table 2). This could be attributed to the fact that AgMUC1 protein is naturally membrane based in the mosquito midgut and is probably presented differently from the secretory circumsporozoite protein of the malaria parasite. The membrane based AgMUC1 antigen in this study is being presented as a secreted protein from the transfected cells and this may affect the uptake and processing of this protein by antigen presenting cells and ultimately influence the type and concentration of antibody produced. This same phenomenon was observed when different components of the merozoite specific protein 1 were administered separately (Cavanagh et al., 2001) and also when a plasmid encoding a rabies protein was administered mixed with GM-CSF plasmid with resultant increase in anti-rabies antibody production (Xiang & Ertl, 1995). When GM-CSF plasmid mixed with a plasmid encoding influenza nucleoprotein were co-administered, enhanced CTL responses as opposed to antibody responses were observed (Iwasaki et al., 1997) while when GM-CSF plasmid mixed with plasmids encoding proteins from HIV-1 were co-administered increased antibody production as well as T cell proliferation were observed (Kim et al., 1997). In this study, Plasmid GM-CSF administered mixed with AgMUC1 cDNA did not result into a striking increase in anti-mucin antibody production (Table 2).

Following co administration of AgMUC1 cDNA with IL-12 cDNA, highly significant differences were observed between the daily cumulative proportions of the blood fed mosquitoes surviving to day 7 post blood feeding when fed on AgMUC1/IL-12 cDNA immunized mice compared to those fed on empty vector/IL-12 cDNA immunized mice (Chisq = 39.1, on 1 degree of freedom, *p = 4.06e-10;* Figure 5.3). IL-12 is a cytokine that has been reported to promote cell mediated immune responses by stimulating the proliferation of activated T lymphocytes, enhancing IFN-γ secretion and the lytic activity by NK cells and CD8+ T-cells (Stern et al., 1996). In the AgMUC1/IL-12 cDNA immunized mice, it is unlikely that the cytotoxic mechanism that promotes the mosquitocidal effect observed is due to CD8+ T cell cytotoxicity. CD8+ T cell mediated cytotoxicity requires the presence of MHC class 1 molecules on the target cells. There are no reports that indicate that these molecules are expressed on the epithelial cells of the blood fed mosquito midgut.

Anti-mucin antibodies present in the blood meal from the AgMUC1/IL-12 cDNA immunized mice likely attach onto the epithelium of the blood fed mosquito midgut. CD8+ T-cells do not have receptors for these attached antibodies hence are most likely not involved in the subsequent events. Thus the only other cytotoxic cells in the blood that have Fc receptors are the NK cells and the macrophages, both of which are activated by IFN-γ (Janeway & Travers, 2001). Since the cytotoxic effects of activated macrophages are effective after phagocytosis, it is unlikely that these cells would play a significant role in killing the blood fed mosquitoes. The furthest they can exert their effects would be through blockage of the channels through which nutrients are absorbed into the mosquito body system which could then lead to mosquito death.

In the murine system, IL-4 preferentially induces switching to IgG1 antibody subtype production while inhibiting the production of IgG2a and IgG3 antibody subtypes (Banchereau et al., 1991). IFN-γ induces switching to IgG2a and IgG3 antibody production while inhibiting IgG1 antibody production (Schijns et al., 1994). In the AgMUC1 cDNA immunized mice, IL-4 was undetectable while increased levels of IFN-γ were observed (Table 3). The IgG antibody subtype produced was IgG1 and a negative correlation with the daily cumulative survival of the blood fed mosquitoes surviving to day 7 (Table 2). In the AgMUC1/IL-12 cDNA group, increased levels of IL-4 and IFN-γ and lower levels of IL-5 were observed compared to the other immunization groups (Table 3). In this group, IL-2 and TNF-α levels were same in both experimental and control groups. IL-12 is known to enhance IFN-γ production while down regulating IL-5 secretion (Chan et al., 1991). In the AgMUC1/GM-CSF cDNA group lower levels of IFN-γ (2020 pg/ml) compared to the other groups are observed. All the other cytokine levels are same in the experimental and control groups. In the AgMUC1 / mucin protein boost group, comparatively lower levels of IL-2, IL-4 and IFN-γ with slightly increased levels of IL-5 were detected in the mucin protein stimulated spleen cells. The predominant IgG antibody subtype produced in all these groups was IgG1 which demonstrated negative correlation with the daily cumulative survival of the blood fed mosquitoes surviving to day 7 in all the groups (Table 2) meaning that this could be the antibody isotype responsible for mosquito death.

In correlating anti-mucin antibody levels to mosquito survival, there was no correlation observed between the daily cumulative survival of the mosquitoes fed on the AgMUC1 / mucin protein immunized mice blood and the antibody levels in these mice as all correlations were < 0.5. This is despite the fact that antibody levels were greatest in this group of mice. Elsewhere, high antibody levels following immunization of rabbits with extracts of *An. stephensi* midgut also showed no statistically significant effect on mortality and longevity of blood feeding mosquitoes (Suneja et al., 2003). In another study, anti-mosquito antibodies produced in mice, inoculated with mosquito homogenates showed significant increase in mortality correlated to both the titer and specificity of the anti-mosquito antibodies detected (Hatfield, 1988). In these studies however the antibodies responsible for these effects were not isotyped. When anti-mucin IgG1 antibodies are specifically correlated with the daily cumulative proportions of the blood fed mosquitoes surviving to day 7, a negative correlation is observed in all the immunization groups ($r > -0.5$; Table 2) showing that it could be the anti-mucin IgG1 antibody subtype that is effective in killing mosquitoes fed on AgMUC1 cDNA immunized mice. When pooled mice sera containing anti-mucin IgG1 antibody subtype was fed on groups of mosquitoes in an *in vitro* membrane feeding system in the presence and absence of complement, no significant differences were observed in the daily cumulative survival of the blood fed mosquitoes surviving to day 7 between the groups of mosquitoes fed to these antibodies in the presence and absence of complement (Chisq = 2.6, on 1 degree of freedom, p = 0.111). Complement independent killing has also been reported following immunization of New Zealand rabbits with *An. gambiae* midguts, which elicited high antibody titers (Noden et al., 1995). High antibody titers following immunization with mosquito midgut extracts have been consistently associated with reduced fecundity in blood feeding mosquitoes (Srikrishnaraj et al., 1993; Suneja et al., 2003; Gakhar et al., 2005). Using only one midgut membrane expressed protein, AgMUC1, as opposed to the use of whole mosquito midgut homogenates, reduced fecundity was not observed in the mosquitoes fed on the AgMUC1 cDNA immunized mice (Foy et al., 2003). Thus it is possible that there could be another migut protein present in the mosquito midgut homogenates, as opposed to the mucin protein, that is responsible for the reduced fecundity.

Thus from this study, we conclude that mortality in mosquitoes fed on AgMuc1 cDNA immunized mice is enhanced when co-administered with IL-12 cDNA. The immune factors responsible for mosquito killing are most likely IgG1 antibody-mediated. We hypothesize that the effector mechanisms of this antibody leading to mosquito death are NK cell mediated antibody dependent cellular cytotoxicity (ADCC). The immunostimulatory effects of IL-12 might probably be involving both antibody- and cell-mediated immune responses. In such a case, the incorporation of this gene into the development of a multifactorial malaria vaccine targeting both the malaria parasite and its mosquito vector could accelerate the search for a vaccine that would reduce disease burden and mosquito density in malaria endemic communities, especially in Africa. With the biological effects of the AgMUC1/IL-12 cDNA immunization strategy being attained day 2 post blood feeding as demonstrated in this study (Table 1) and the *An. gambiae* reported to be taking 2-3 blood meals per gonotrophic cycle (Colluzi, 1992) and 4-12 blood meals to be able to transmit the infective sporozoite (Killeen et al., 2000), the chances of the mosquito surviving to transmit the infective sporozoites during it's next blood meal are completely minimized. Thus vaccine coverage and efficacy with such a multifactorial malaria vaccine strategy need not be necessarily high in endemic malaria transmission areas in order to alleviate disease burden (Foy, Killeen, Magalhaes, & Beier, 2002).

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