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Adenovectors induce functional antibodies capable of potent inhibition of blood stage malaria parasite growth

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ABSTRACT

An effective malaria vaccine remains a global health priority. Recombinant adenoviruses are a promising vaccine platform, and *Plasmodium falciparum* apical membrane antigen 1 (AMA1) and merozoite surface protein 1–42 ($MSP1_{42}$) are leading blood stage vaccine candidates. We evaluated the importance of surface antigen localization and glycosylation on the immunogenicity of adenovector delivered AMA1 and $MSP1_{42}$ and assessed the ability of these vaccines to induce functional antibody responses capable of inhibiting parasite growth *in vitro*. Adenovector delivery induced unprecedented levels of biologically active antibodies in rabbits as indicated by the parasite growth inhibition assay. These responses were as potent as published results using any other vaccine system, including recombinant protein in adjuvant. The cell surface associated and glycosylated forms of AMA1 and $MSP1_{42}$ elicited 99% and 60% inhibition of parasite growth, respectively. Antigens that were expressed at the cell surface and glycosylated were much better than intracellular antigens at inducing antibody responses. Good T cell responses were observed for all forms of AMA1 and $MSP1_{42}$. Antigen-specific antibody responses, but typically not T cell responses, were boosted by a second administration of adenovector. These data highlight the importance of rational vaccine design and support the advancement of adenovector delivery technology for a malaria vaccine.

1. Introduction

Malaria is the most devastating parasitic disease affecting humans. Each year there are 300–500 million new infections and greater than one million deaths [1]. Antibodies against blood stage antigens are thought to be important in immunity to malaria, since passive transfer of purified immunoglobulin from individuals with lifelong exposure to endemic malaria results in a marked decrease in parasitemia and resolution of symptoms in the recipients [2]. Parasite proteins expressed on the surface of infected erythrocytes and merozoites and in merozoite apical organelles, including the merozoite surface protein 1 (MSP1) and the apical membrane antigen 1 (AMA1), are considered high priority antigens for blood stage vaccine development [3].

AMA1 [reviewed in [4]] is a 72 kDa protein that is located in the apical microneme organelles and then on the surface of the merozoite [5] and is involved in erythrocyte invasion [6]. AMA1 subunit vaccines can protect against experimental challenge in animal models and AMA1 antibodies induced by natural exposure or experimental immunization with candidate subunit vaccines can block invasion of human erythrocytes by *Plasmodium* parasites in an *in vitro* growth inhibitory assay (GIA) [7–10]. AMA1 has been identified in *Plasmodium* sporozoites [11] suggesting that T cell responses specific for AMA1 may also function in protection.

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MSP1 is a large protein that is proteolytically processed into at least four distinct fragments, of which the C-terminal 42 kDa fragment ($MSP1_{42}$) is of particular interest [12]. $MSP1_{42}$ contains a C-terminal 19 kDa fragment ($MSP1_{19}$) that remains attached to the merozoite membrane through a glycosylphosphatidylinositol (GPI) anchor during invasion as well as N-terminal T cell epitopes. Antibodies that target the 19 kDa fragment are associated with *Plasmodium falciparum* growth inhibition *in vitro* and with reduced burden of malaria disease in endemic populations in some epidemiologic studies [13]. Immunization with MSP1 fragments can protect mice against *Plasmodium yoelii* challenge [14] and monkeys against *P. falciparum* challenge [15,16]. MSP1, like AMA1, is expressed in *Plasmodium*-infected hepatocytes [17–19] but its expression has not been identified in sporozoites.

Adenovectors induce strong and protective antibody- and T cell-mediated immune responses in multiple infectious disease systems [20,21], including malaria [22–24] and in multiple animal models including mice and non-human primates. Adenovirus serotype 5 (Ad5) vectors are currently being evaluated in clinical trials for vaccines against HIV [25–27], tuberculosis, and malaria. CD4+ T cell, CD8+ T cell, and antibody responses have been induced in a majority of volunteers by Ad5-based HIV vaccines [25,26]. Since studies in animal models demonstrate that CD8+ T cells are critical effectors in pre-erythrocytic stage immunity directed against the liver stage of the parasite life cycle [26a], these findings suggest that adenovectors may be able to induce the requisite immune responses for protection against *P. falciparum* malaria.

Induction of strong antibody responses against blood stage antigens is likely required for an effective vaccine targeting the blood stage of the malaria parasite, although T cell responses may also play a role. The way an antigen is presented to the immune system impacts the capacity of that antigen to induce potent antibody responses. For example, secretion or cell surface expression as opposed to intracellular expression can induce a more robust antibody response [28,29]. In contrast, antigen secretion is not a prerequisite for the induction of T cell responses [30,31]. Another factor that could influence the humoral response is the presence or absence of glycosylation sites. *P. falciparum* parasites do not contain significant amounts of N- and O-linked carbohydrates [32]. However, when delivered to eukaryotic cells with viral- or plasmid-based delivery systems, many *P. falciparum* proteins, including AMA1 and MSP1, are expected to be glycosylated. Glycosylation may affect the structure of the antigen or mask potential antigenic epitopes and could interfere with the immunogenicity of *Plasmodium* antigens delivered by adenovectors. For example, in one study, Aotus monkeys were protected against *P. falciparum* blood stage challenge by immunization with a non-glycosylated form of $MSP1_{42}$ produced in mouse milk but not by immunization with a glycosylated version (also milk-derived) [33]. However, other studies with $MSP1_{42}$, AMA1, and PfEBA175 subunit protein vaccines and DNA-AMA1 and DNA-$MSP1_{42}$ vectors have indicated that glycosylated proteins can be effective vaccines [12,33,34]. Therefore, we have evaluated the effect of antigen cellular localization and glycosylation on the immunogenicity of *P. falciparum* AMA1 and $MSP1_{42}$ antigens in the context of an Ad5-based vaccine. Antigen-specific T cell and antibody responses were assessed in mice and antibody titers and functional antibody responses were assessed in rabbits.

2. Materials and methods

2.1. Cells and media

293-ORF6 is a GenVec proprietary cell line derived from 293 cells, a human embryonic kidney cell line. It contains adenovirus type 5 early region 4 open reading frame 6 (E4-ORF6) and complements for both the E1 and the E4 adenovirus functions [35,36]. A549 cells are human alveolar basal epithelial cells obtained from the American Type Culture Collection (Manassas, VA) and were used for *in vitro* antigen analysis. 293-ORF6 and A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). A20.2J (ATCC clone HB-98) is a mouse B cell line that was obtained from ATCC and maintained in RPMI-1640 medium supplemented with 20% FBS and 1% glutamine.

2.2. Adenovirus vector construction

Adenovirus vectors expressing the blood stage antigens AMA1 and $MSP1_{42}$ were constructed using shuttle vectors as previously described [37]. Antigen genes were built into expression cassettes located in either the E1 or E4 regions of an E1-, partial E3-, E4-deleted adenovirus serotype 5 vector. These vectors were constructed and produced in 293-ORF6 cells. Viruses were purified from suspension cells between 2 and 3 days after infection by three freeze–thaw cycles followed by benzonase digestion and three successive bandings on CsCl gradients. Total particle unit titer was determined by optical absorbance.

2.3. Animals

Female 6–8 weeks old BALB/c AnNCr mice were purchased from the National Cancer Institute (Frederick, MD). All rabbit studies reported herein were conducted under contract at Spring Valley (Woodbine, MD) in 1.5–2.5 kg (~6 weeks old), female, New Zealand white rabbits purchased from Harlan (Indianapolis, IN).

2.4. Immunizations

BALB/c mice ($n = 6$/group) were immunized by bilateral injections into tibialis anterior muscles with 1×10^8 particle units (pu) of antigen expressing adenovirus vector in a total volume of 0.1 ml using a 29.5*G* needle. Adenovirus vectors without any inserted gene (AdNull) and unimmunized naive mice were used as negative controls in all studies. NZW rabbits ($n = 6$/group) were immunized by two 0.5 ml injections into the right quadricep muscles with 1×10^{10} particle units of antigen expressing adenovirus vector using a 26*G* needle. For T cell studies, spleen cells from immunized or control mice were harvested for use in IFN-γ ELIspot assays ($n = 6$ mice/group, assayed in pools) or intracellular cytokine staining assays ($n = 6$ mice/group, assayed individually) at 2 or 6 weeks after the final immunization. For antibody studies, sera from immunized or control mice ($n = 6$ mice/group, assayed individually) were collected 2 or 6 weeks after each immunization.

2.5. Immunofluorescence assay

A549 cells in a 12-well plate were infected at 70% confluence with various adenovectors at a MOI of 200 pu/cell for 1 h and then overlayed with DMEM medium containing 5% FBS. Twenty-four hours later, cells were washed 3 times for 5 min each with PBS and fixed with 4% paraformaldehyde (1 ml) for 30 min at room temperature. Cells were washed with PBS again and incubated for 2 h at 37 °C with primary antibody (1:200) in PBS containing 0.5% BSA ± 0.1% saponin for cell permeablization. Cells were again washed 3 times with PBS and incubated for 1 h at 37 °C with secondary antibody conjugated with fluorescein isothiocyanate (FITC) (1:200) in PBS containing 0.5% BSA. Cells were viewed using a Nikon Labophot II microscope and images were acquired using a Spot RT digital camera. The 4G2 monoclonal antibody was used for analysis