EBioMedicine 9 (2016) 207–216

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Complement and Antibody-mediated Enhancement of Red Blood Cell Invasion and Growth of Malaria Parasites

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ARTICLE INFO

Article history:
Received 3 February 2016
Received in revised form 3 May 2016
Accepted 12 May 2016
Available online 14 May 2016

Keywords:
Malaria
Complement
Red blood cells
Merozoites
CR1

ABSTRACT

Plasmodium falciparum malaria is a deadly pathogen. The invasion of red blood cells (RBCs) by merozoites is a target for vaccine development. Although anti-merozoite antibodies can block invasion in vitro, there is no efficacy in vivo. To explain this discrepancy we hypothesized that complement activation could enhance RBC invasion by binding to the complement receptor 1 (CR1). Here we show that a monoclonal antibody directed against the merozoite and human polyclonal IgG from merozoite vaccine recipients enhanced RBC invasion in a complement-dependent manner and that soluble CR1 inhibited this enhancement. Sialic acid-independent strains, that presumably are able to bind to CR1 via a native ligand, showed less complement-dependent enhancement of RBC invasion than sialic acid-dependent strains that do not utilize native CR1 ligands. Confocal fluorescent microscopy revealed that complement-dependent invasion resulted in aggregation of CR1 at the RBC surface in contact with the merozoite. Finally, total anti-*P. berghei* IgG enhanced parasite growth and C3 deficiency decreased parasite growth in mice. These results demonstrate, contrary to current views, that complement activation in conjunction with antibodies can paradoxically aid parasites invade RBCs and should be considered in future design and testing of merozoite vaccines.

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1. Introduction

Malaria, a mosquito-borne infectious disease caused by eukaryotic intracellular protists of the genus *Plasmodium*, kills close to one million people worldwide each year, predominantly children under 5 years of age (Murray et al., 2012; World Health Organization, 2013). Of the five species of *Plasmodium* that can infect humans, infection with *Plasmodium falciparum* accounts for the vast majority of deaths worldwide. *Plasmodium*'s complex life cycle involves invasion of hepatocytes and red blood cells (RBCs); however, the clinical symptoms arise from the invasion of RBCs by the asexual blood stage parasite. Antibodies are thought to play an important role in natural immunity as demonstrated by the reduction in parasitemia and clinical symptoms in *P. falciparum*-infected individuals following passive transfer of immunoglobulins from semi-immune donors (Cohen et al., 1961; McGregor, 1964a; Sabchareon et al., 1991). However, the effector mechanisms are poorly understood.

Development of a vaccine to block RBC invasion has proven to be an elusive goal. Much of the effort has been focused on the merozoite surface protein 1 (MSP1) and the apical membrane antigen 1 (AMA1). Multiple preclinical vaccine studies have demonstrated, using growth inhibition assays (GIA), that antibodies targeting MSP1 and AMA1 of *P. falciparum* have in vitro RBC invasion and growth inhibitory activity (Angov et al., 2003; Chang et al., 1992; Kennedy et al., 2002). In addition, some degree of protective immunity has been seen in some animal models (Darko et al., 2005; Singh et al., 2003; Singh et al., 2006). Unfortunately, to date, these studies have not translated into in vivo efficacy in human vaccine trials (Ogutu et al., 2009; Sagara et al., 2009; Spring et al., 2009). Thus, GIA is a poor predictor of blood stage protective immune responses despite the fact that antibodies do inhibit RBC invasion. The reasons for this discrepancy are unknown.

One possible explanation for this discrepancy came to light as the result of the discovery that the complement receptor 1 (CR1) is a sialic acid (SA)-independent receptor for *P. falciparum* (Spadafora et al., 2010; Tham et al., 2010). The complement system is part of the innate immune response and is an important effector arm of humoral immunity. It can be activated via three main pathways: the classical pathway (CP); the lectin pathway (LP); and the alternative pathway (AP)

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http://dx.doi.org/10.1016/j.ebiom.2016.05.015
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(Ricklin et al., 2010). Once activated, the complement system induces the formation of opsonins (C3b, C4b) that promote phagocytosis, induce lysis by formation of the terminal complement complex (TCC), and promote an inflammatory response (Ricklin et al., 2010). Once bound to the pathogen, surface C3b and C4b serve as ligands for CR1, which is present on RBCs as well as most leukocytes (Fearon, 1980; Tas et al., 1999). CR1 also binds complement factors C1q and mannan-binding lectin (MBL) (Ghiran et al., 2000; Tas et al., 1999).

We hypothesize that *P. falciparum* is capable of exploiting the opsonizing qualities of complement deposition on the merozoite surface which will allow it to bind to CR1 and invade via this invasion pathway. If we are correct, complement activation could negate the inhibitory activity of anti-merozoite neutralizing antibodies generated post vaccination or during natural infection.

2. Materials and Methods

2.1. Parasites, Parasite Culture, and RBC Treatment

SA-independent strains (7G8, 3D7, HB3, and Dd2NM) were obtained from the Walter Reed Army Institute of Research. SA-dependent strains (FVO, Camp, Dd2, and FCR3) were obtained from the Malaria Research and Reference Reagent Resource Center (BEI Resources, Manassas, VA). Parasite cultures were maintained at 1–4% hematocrit (Hct) in O + blood with 10% heat-inactivated (HI) plasma in RPMI 1640 Medium (Sigma-Aldrich, St. Louis, MO) with 25 μg/ml gentamicin, 20 μg/ml hypoxanthine, and 7.5% w/v NaHCO3 (complete media) in malaria gas (5% O_2, 5% CO_2, and 95% N) at 37 °C. Cultures were synchronized twice a week by 5% sorbitol lysis (Lambros and Vanderberg, 1979). Neuraminidase treatment of RBCs was carried out as described (Spadafora et al., 2010).

2.2. Sera, Complement Factors, and CH50/AH50 Assay

Non-hemolyzed whole blood was collected with a 21 gauge needle from two O + volunteers into glass tubes without additives (Becton Dickinson, Franklin Lakes, NJ) and allowed to clot at room temperature for 50 min. The samples were centrifuged at 1300 ×*g* for 15 min and the serum was removed and re-centrifuged for 5 min again to pellet any residual RBCs or clot particles. The serum was aliquoted and stored at −80 °C. Serum was used fresh (FS) or after heat inactivation (HIS) at 56 °C for 30 min in 200 μl aliquots in 1.5 ml polypropylene microcentrifuge tubes (Denville Scientific, South Plainfield, NJ). Serum C2 and Factor B (fB) were selectively inactivated by incubation of 200 μl serum aliquots in 1.5 ml microcentrifuge tubes in a 56 °C water bath for 3 min with constant mixing (Araujo et al., 1991). Purified complement factors (C1q, C2, C3, C4) and selectively depleted or inactivated sera were obtained commercially (CompTech, Tyler, TX). 50% CP and AP complement hemolytic (CH50 and AH50) assays were performed using the standard methods described in the literature (Morgan, 2000).

2.3. In Vitro Invasion Assays

Invasion assays were carried out in triplicate wells of a 96-well plate containing sorbitol-synchronized cultures of late trophozoites or schizonts at 0.5% to 2% parasitemia in 2–4% hematocrit. RBCs had a mean CR1 expression of 600 molecules per RBC as measured by flow cytometry (Spadafora et al., 2010). Inhouse prepared serum (FS or HIS) was always autologous to the RBCs used in the assays. Complement-depleted or C3/C4-inactivated sera were always commercially-obtained (CompTech, TX) and came from pooled donor blood, and was thus, heterologous to the RBCs in the assays. Sera were added to a final concentration of 10%. The cyclical C3 inhibitor peptide compstatin (NH3-IIe-Cys-Val-Val-Gln-Asp-Trp-Gly-His-His-Arg-Cys-Thr-COOH) (Tocris Bioscience, Bristol, UK) (Mastellos et al., 2015), was used to determine the effect of blocking C3. A peptide derived from the linearized and scrambled sequence of compstatin without cysteins (NH3-Arg-Thr-Ala-Trp-Gln-His-Asp-Ala-Ile-His-Val-Gly-Val-COOH) was synthesized and used as a control. sCR1 was used as an inhibitor (Celldex Therapeutics, Hampton, NH) and fetuin (Sigma-Aldrich, St. Louis, MO), an inert glycoprotein with no complement activity, was used as negative control protein where appropriate. Antibodies were added at different concentrations. Mouse monoclonal antibody mAb5.2 was raised against the 19 kDa subunit of the merozoite surface protein 1 ($MSP1_{19}$) (Siddiqui et al., 1986) and was purified from cultured hybridoma by protein A/G chromatography (Thermo Fisher Scientific, Rockford, IL). Mouse IgG2b Clone eBMG2b (eBioscience, San Diego, CA) was used as isotype control. IgG from individuals vaccinated with $MSP1_{42}$ (Otsyula et al., 2013), comprising the C-terminal 42-kDa portion of the FVO variant of *P. falciparum* MSP1, was purified by protein A/G chromatography. The plate was placed in a gas-impermeable heat-sealable bag and inflated with malaria culture gas (Haynes et al., 2002). As an alternative procedure we used filter-purified merozoites as described by Boyle et al. (2010, 2015) with the exception that late trophozoite/schizonts were enriched using a Percoll gradient (Moll et al., 2008). After 20 to 24 h, 5 μl aliquots of individual wells were added to 100 μl 5 μg/ml Hoechst 33342 (Life Technologies, Grand Island, NY) in PBS containing 2% paraformaldehyde (Sigma-Aldrich). At least 100,000 RBCs were acquired for each sample. Acquisition was done using a LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with a violet laser and analysis was performed using FCS Express (De novo Software, Glendale, CA). RBCs with Hoechst-positive ring stage parasitemia (early trophozoite) were used as endpoint for 24-h invasion assays. The background staining of an uninfected RBC sample was subtracted. The majority of the experiments were repeated 2–3 times.

2.4. Merozoite Attachment Assay

Unless otherwise stated, all the centrifugation steps were at 770 ×*g* for 5 min at room temperature (RT). Attachment assays were carried out as invasion assays except that highly synchronous late stage parasite cultures were incubated with 10 μg/ml of leupeptin (Sigma-Aldrich) for 6 to 8 h followed by three washes with RPMI 1640. The hematocrit was adjusted to 2% by the addition of fresh RBCs. The final culture medium also contained 2 μM cytochalasin D (Sigma-Aldrich) and 10% complement deficient or reconstituted serum in the presence or absence of mouse mAb5.2 or IgG2b. The cultures were incubated again for 3–4 h at 37 °C in malaria gas followed by centrifugation at 400 ×*g* for 1 min at 4 °C and two washes with 100 μl of 1% BSA/PBS blocking buffer. mAb5.2 (2 mg/ml), if not added previously, and goat polyclonal anti-C3 (MP Biomedicals, Santa Ana, CA) antibodies diluted 1:33 in blocking buffer were added for 30 min at 4 °C. Following three washes with blocking buffer, the pellets were resuspended in 1:100 dilution of donkey anti-goat IgG-PerCP R&D Systems, Minneapolis, MN) and goat anti-mouse DyLight 488 (KPL, Gaithersburg, MD) in blocking buffer and incubated for 30 min at 4 °C. After an additional three washes with blocking buffer the pellets were resuspended in 2% paraformaldehyde/PBS with 5 μg/ml Hoechst 33342 (Life Technologies) solution. Acquisition by flow cytometry was carried out as above.

2.5. Confocal Microscopy of Merozoite Attachment

For confocal microscopy of the interaction of merozoites with RBCs the culture conditions were the same as for attachment assays. After incubation, the pellets were resuspended in 1% BSA/PBS blocking buffer containing 130 μg/ml mouse anti-MSP1 mAb5.2 (if not present during the assay), 190 μg/ml of chicken polyclonal anti-CR1 (Gallus Immunotech, ON, Canada), and 30 μg/ml of mouse monoclonal 1H8 anti-C3 IgG1 (Kerafast, Boston, MA) and incubated for 30 min at 4 °C. The specificity of each primary antibody was verified by the use of negative control antibodies. After three washes with blocking buffer, the pellets were resuspended in 100 μl of blocking buffer containing 1:500

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