Anti-sporozoite antibodies

M.R. Hollingdale[1]

Development of anti-sporozoite vaccines based on CS (circumsporozoite) proteins has preceded a thorough understanding of the basic biology of sporozoite–hepatic cell interactions. Investigation of these interactions can only serve to further refine the existing sporozoite vaccines, as well as provide a rationale for the design of other vaccine candidates.

Introduction

Malarial sporozoites injected into the blood stream by the bite of an infected mosquito are rapidly cleared from the circulation and invade liver parenchymal cells. There, the sporozoites transform into exoerythrocytic (EE) parasites, and by asexual schizogony develop into thousands of EE merozoites which, after rupture of the infected hepatocytes, invade the red blood cells. Sera of individuals living in malaria-endemic areas, particularly adults, react with sporozoites by IFA (*1*), suggesting that anti-sporozoite antibodies may protect against sporozoite infection. The experimental induction of protective anti-sporozoite antibodies by vaccines has been the subject of intense research. Our understanding of how anti-sporozoite antibodies may confer protection, however, is still limited, and more knowledge of the basic biology of sporozoite–liver cell interactions is required.

Sporozoite–liver interactions

Sporozoites enter the blood stream by the bite of a mosquito, which repeatedly probes the skin tissue and releases sporozoites before ingesting its blood meal. Little information is available on the immunological interaction of sporozoites with antigen presenting cells (APC) or with immune effector mechanisms, humoral or cellular, present in the skin. It is probable that host immune responses may differ when sporozoite infection is by a bite or experimental intravenous inoculation.

Sporozoites are rapidly trapped in several organs including the spleen, and over 95% of injected sporozoites were found to be retained in the liver after direct perfusion (*2, 3*). Considerable discussion, reviewed by Meis & Verhave (*4*), has centred around the actual mechanism by which sporozoites passage from the liver sinusoid into the hepatocytes. Liver sinusoids are lined by capillary endothelial cells and Kupffer (macrophage) cells, in direct contact with the blood stream, whereas parenchymal cells, and the more rare pit cells, which may have natural killer cell activity, are in contact with the plasma in the space of Disse. The endothelial cells possess thin extended processes containing small pores, called fenestrae, grouped into sieve plates, and are highly endocytic.

It is still unclear whether the sporozoites penetrate directly into the space of Disse through the fenestrae (*5*), or by first passing through the Kupffer cells (*6*). Careful fixation of the liver by perfusion has revealed that the diameter of the fenestrae is about 0.1 μm (*7, 8*), too small to allow direct passage of sporozoites with a diameter of 1 μm. In rats injected with sporozoites, the Kupffer cells rapidly take up the sporozoites (*5, 6, 9*), which are found within membrane-bound vacuoles (*9*) and appeared to be in a cytoplasmic projection in the space of Disse (*9*). The vacuole membranes do not fuse with the lysozomes, and the sporozoites remained morphologically intact for at least one hour (*9*). *In vitro* studies also demonstrated that sporozoites survive intact in mouse peritoneal macrophages (*10*), and did not increase the production of free oxygen radicals (*11*), whereas heat-inactivated or trypsinized sporozoites induced the oxidative response (*11*). Non-viable or non-infective sporozoites were retained as effectively in the liver as the viable, infectious sporozoites (*2, 3*), suggesting that the sporozoites are taken up by the Kupffer cells by active phagocytosis. Viruses (*12*) and several species of *Eimeria* (*13*) have also been shown to passage through the macrophages. While these results are persuasive, penetration through the fenestrae or directly through endothelial cells cannot be excluded. *In vitro* studies have also shown that sporozoites are able to invade hepatic cells directly without macrophage involvement. However, the role of Kupffer cells in both facilitating hepatic infection and generating a protective immune response as APCs may be related. Irradiated or live sporozoites that invade hepatocytes are highly immunogenic, whereas heat-killed or dis-

[1] Biomedical Research Institute, 12111 Parklawn Drive, Rockville, MD 20852, USA.

rupted sporozoite antigens elicit a weak, variable immune response (*14*).

Sporozoite–hepatic cell interactions in vitro

EE parasites of rodent, simian and human malarias have been cultured *in vitro* in a variety of cells, including primary hepatocytes. The human hepatoma cell line, HepG2-A16, is metabolically and ultrastructurally closely similar to isolated primary hepatocytes, and the rodent malaria *Plasmodium berghei* and the human malaria *P. vivax* both invade and develop into EE parasites in these cells (*15, 16*). An important difference is that *P. falciparum* sporozoites invade both primary hepatocytes (*17*) and HepG2-A16 cells (*18*), but only develop into EE parasites in primary hepatocytes (*17*), and these differences have been exploited to study the molecular events of invasion and EE development.

Ultrastructural studies have shown that sporozoites attach to hepatic cells at the apical end and invade by invagination of the hepatic cell membrane, which forms the parasitophorous vacuole membrane enclosing the sporozoite (*19*). Monoclonal antibodies (MAb) to the repeat region of circumsporozoite (CS) proteins completely block sporozoite attachment and invasion of hepatic cells *in vitro* (*18, 20*), suggesting that repeat regions are the ligands that recognize specific hepatic receptor(s). However, studies with radiolabelled synthetic peptides have suggested that the conserved CS region, N1, immediately adjacent *N*-terminal to the repeat region, showed specific and saturable binding to HepG2-A16 cells (*21*). Binding of repeat region peptides, although non-saturable, was significant, and it was suggested that the general affinity of the repeat region for cell membranes may non-specifically mediate sporozoite attachment (*21*). Purified rabbit IgG against N1 strongly blocked *P. falciparum* sporozoite invasion of HepG2-A16 cells, suggesting that the N1 region plays a major role in sporozoite invasion of hepatocytes. Further studies (Aley & Hollingdale, unpublished observations) demonstrated that the binding site of N1 was contained within the sequence KLKQP, which is present in CS proteins of all species that invade HepG2-A16 cells, and varies as KLNQP in *P. yoelii* which does not invade.

The probable receptor for N1 on HepG2-A16 cells was identified by cross-linking studies as two proteins of 55 and 35 kDa (*21*). Antibodies to KLKQP elicited by KLKQP-proteosome vaccines (*22*) blocked invasion of *P. falciparum* sporozoites of HepG2-A16 cells, but not invasion of *P. berghei* sporozoites. Since anti-KLKQP antibodies reacted with *P. berghei* sporozoites by IFA (*22*), it is possible that either KLKQP is not involved in *P. berghei* sporozoite invasion of HepG2-A16 cells, or that molecular events other than KLKQP-HepG2-A16 interactions, that lead to development of *P. berghei* EE parasites in HepG2-A16 cells overcome the anti-KLKQP antibodies. Thus, it is probable that *P. falciparum* sporozoite invasion of HepG2-A16 cells is initially similar to that of *P. berghei* sporozoite invasion, but that additional molecular events required for EE development occur only with *P. berghei* sporozoites.

Cross-linking studies using *P. falciparum* sporozoites and primary human hepatocytes revealed two receptors of 55 and 20 kDa (*23*). Monospecific sera to the hepatocyte 55 kDa receptor did not react in Western blots with HepG2-A16 cells, and blocked *P. falciparum* invasion of primary hepatocytes but not HepG2-A16 cells. Preliminary results in which the purified hepatocyte 55 kDa receptor was cross-linked to *P. falciparum* sporozoites identified a 16 kDa non-CS protein (sporozoite hepatocytic binding antigen, SHEBA), which may thus also represent a *P. falciparum* sporozoite ligand for hepatocyte invasion. Thus, these receptors are present on primary human hepatocytes, but not HepG2-A16 hepatoma cells, and could represent sporozoite–hepatocyte interactions in addition to N1 interactions that lead to successful EE development. Experiments are in progress to further define sporozoite ligand–hepatocyte interactions, not least to characterize additional sporozoite vaccine candidates.

A fundamental question is whether anti-sporozoite antibodies are protective, and how such protection is conferred. Clearly, irradiated sporozoites elicit protection in man (*24*) and rodents (*25*). Passive serum transfer experiments in mice have not demonstrated that irradiated sporozoites elicit protective antibodies, and indeed antibody-independent mechanisms may themselves be protective (*26, 27*). However, passive transfer of MAbs to *P. berghei* CS proteins, and mouse anti-*P. berghei* CS peptide antibodies, have protected mice to sporozoite challenge (*28, 29*), and it is possible that the failure of passively transferred anti-sporozoite antibodies, even if partially purified on protein A columns (*29*), may reflect low concentration rather than absence of activity. However, the question of whether naturally acquired anti-sporozoite antibodies are protective is more controversial. Anti-*P. falciparum* sporozoite antibodies in sera from malaria-endemic areas have been detected by IFA (*1*), and react by ELISA with *P. falciparum* CS repeat peptides (*30–33*), and the level of acquired anti-sporozoite antibodies rose with age, suggesting their protective capacity. In a study in Kenya (*34*), where adults were drug-treated to clear malaria infection, no correlation was found between ELISA activity to CS protein and protection to natural transmission. Since

anti-malarial immune mechanisms other than those against sporozoites would presumably also be acquired by these individuals, these results suggest the failure of anti-sporozoite assays to predict protection rather than the failure of anti-sporozoite antibodies to protect.

Inhibition of sporozoite invasion (ISI)

Sera from human volunteers immunized with irradiated *P. falciparum* or *P. vivax* sporozoites, and protected to homologous sporozoite challenge (*24*) inhibit *P. falciparum* or *P. vivax* sporozoite invasion (ISI) of HepG2-A16 cells (*18*), suggesting that ISI is an assay of protective antibodies. Since efficacy of *P. falciparum* sporozoite vaccines can only be tested in man because of the lack of a suitable non-human primate challenge model, the ISI assay has been widely used to measure anti-*P. falciparum* CS antibodies in small animal and human trials. CS vaccines elicited considerable ISI activity in mice and rabbits, but were poorly immunogenic in man. However, in early trials, both the recombinant R32tet32 and synthetic $(NANP)_3$-tetanus toxoid vaccines each protected one of three volunteers to sporozoite challenge (*35, 36*). Since lymphocyte proliferation assays were not correlated with protection, it is likely that protection elicited by these vaccines was purely by antibodies. Similar results were demonstrated in mice immunized with *P. berghei* CS vaccines (*29, 37*). Therefore, whether or not naturally-acquired anti-sporozoite antibodies protect, protective antibodies can be elicited by CS vaccines. Partially purified IgG from the protected volunteers was more active in the ISI assay using *P. falciparum* sporozoites and HepG2-A16 cells than IgG from non-protected volunteers (*38*), suggesting that ISI rather than IFA or ELISA activity may be a better correlate of protection. Therefore, the age-dependent increasing ISI activity seen with sera from malaria-endemic areas (*32, 33*) may also suggest that naturally-acquired anti-sporozoite antibodies are protective, at least in adults. However, the failure of ISI to predict protection in the Kenyan study (*34*) suggests that the ISI assay is correlative with but not predictive of naturally acquired protective antibodies. The ISI assay, however, may have predictive capacity in vaccine trials.

Purified IgG from CS vaccine immunized volunteers blocked *P. falciparum* sporozoite invasion of HepG2-A16 cells, but did not block *P. falciparum* sporozoite invasion of human hepatocytes (*38*). As discussed above, CS proteins probably mediate part of the sporozoite invasion process of both HepG2-A16 cells and primary human hepatocytes. However, other interactions, which do not occur with HepG2-A16 cells, are required for EE development, and may overcome anti-CS antibody activity. But since CS vaccines elicited antibodies in mice that blocked *P. falciparum* sporozoite invasion of HepG2-A16 cells (*35, 36*) and hepatocytes (*20*), and elicited T-helper cell activity, it seems essential that CS vaccines must contain T-helper epitopes recognized by humans in order to elicit antibodies that will effectively block invasion of hepatocytes. Thus, ISI activity with HepG2-A16 cells may be an *in vitro* correlate of protective anti-sporozoite activity, but may in no way represent how such antibodies protect *in vivo*. In fact a danger would be to suppose that an *in vitro* assay should necessarily reflect the actual mechanism which it is either correlative with or predictive of.

If sporozoites passage through Kupffer cells to reach hepatocytes in the space of Disse, it is probable that anti-sporozoite antibodies could confer protection *in vivo* by opsonization of sporozoites by Fc receptors on Kupffer cells, leading to lysosomal fusion and sporozoite destruction. Studies with peritoneal macrophages showed that sporozoites survived intracellularly for extended periods, but when pre-incubated with anti-sporozoite antibodies, the phagocytosed sporozoites were rapidly destroyed (*11*). Thus, effective sporozoite vaccines should elicit antibodies that are active in ISI assays using HepG2-A16 cells and hepatocytes, and such vaccines are likely to contain both CS and other sporozoite antigens.

While development of anti-sporozoite vaccine that elicits protective antibodies is a major goal, several experiments have suggested that such antibodies may have an unwanted impact on sporozoite transmission (*39–42*). *P. falciparum*-infected mosquitos that were immune-fed with anti-sporozoite antibodies during the early stages of sporogony when CS proteins are first synthesized, developed more salivary gland sporozoites than control mosquitos (*39*), and such sporozoites were no longer neutralized in the ISI assay with anti-CS recombinant or synthetic peptide sera from human vaccine trials (*40*). While enhancement of sporozoite density might be a nutritional effect (*41*), antibody-induced qualitative differences in sporozoite susceptibility to CS vaccine-elicited antibodies may present problems if such vaccines are introduced into endemic areas, especially since CS vaccine studies have shown that high levels of anti-CS antibodies protect against only low numbers of sporozoites (*29*). It will be important to determine whether infected mosquitos naturally exposed to anti-sporozoite antibodies develop sporozoites that overcome naturally acquired or vaccine-induced anti-sporozoite immunity.

Whether other *P. falciparum* sporozoite or EE proteins elicit protective antibodies requires further investigation (*42*).

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