Evidence implicating MHC genes in the immunological nonresponsiveness to the *Plasmodium falciparum* CS protein

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The circumsporozoite (CS) protein is a major candiate vaccine antigen for the sporozoite stage of malaria. Both cytotoxic T cells (CTL) and antibody specific for the CS protein are thought to be important in protection. By examining the immune response in mice and humans we have shown that genes mapping to the major histocompatibility complex (MHC) are important for immune responsiveness. F1 mice between high antibody responders and low antibody responders are high antibody responders, suggesting that in this model immune suppressor *genes do not control the immune response. Using synthetic peptides to map epitopes for CTL and helper T cells (which are important for the antibody response) we have shown that the T-cell epitopes are located in the polymorphic region of the protein, and we hypothesize that T cells have indeed selected the variation observed in the CS protein. The success of subunit vaccines will depend on the pattern of variation in different geographical locations, the ability to construct multivalent vaccines containing different variant epitopes from this protein, and on the existence of other sporozoite and liver-stage proteins involved in protection.*

Introduction

Humans and laboratory animals can be immunized to malaria sporozoites (*1–3*). In these experiments, immunization was by the bite of large numbers of irradiated infectious mosquitos or by intravenous injection of irradiated sporozoites. Natural sporozoite immunity, however, may not readily develop in people from endemic regions (*4*). Since the circumsporozoite (CS) protein appears to be a target for protective immunity (see ref. *5* for a recent elegant demonstration), we analysed the cell types responsible for immunity to sporozoites and the immunogenicity of the CS protein in humans and laboratory animals.

Chen and colleagues demonstrated that μ-suppressed mice (which lacked antibodies or circulating B cells) could be protectively immunized with irradiated sporozoites (*6*). Since athymic mice could not be immunized, these data demonstrated that T-cells, in the absence of antibody could protect from viable sporozoite challenge. To ask which subset of T-cells was responsible for antibody-independent immunity, we depleted *Plasmodium yoelii*-immune mice of different T-cell subsets by the parenteral administration of either anti-CD8 or anti-CD4 monoclonal antibodies. Mice depleted of CD8, but not CD4 immune T cells were not protected, suggesting that cytotoxic T lymphocytes (CTL) were responsible for immunity (*7*). Similar findings were reported using the *P. berghei* system (*8*).

Methods and discussion

Although the CS protein is known to be a target for protective antibodies (*9*), it was not known whether it was a target for protective CTL. As a start in addressing this question, we transfected the gene for the CS protein of *P. falciparum* into murine L cells (*10*). We then showed that spleen cells from mice (B10. BR ($H\text{-}2^k$)) immunized with either irradiated *P. falciparum* sporozoites or CS-recombinant vaccinia (kindly provided by Dr B. Moss) contained CS-specific CTL. Using overlapping synthetic peptides, we then mapped the epitope recognized by the CTL to residues 368–390 of the CS protein. We are currently studying the human CTL response to this protein, as well as the murine CTL response to the *P. yoelii* CS protein. However, since human and murine T-cells often recognize similar regions of proteins (*11*), it is likely that the human response to the protein will include this carboxyterminal region recognized by mice. Unfortunately, this region is known to be variant. B10.BR mice appeared to recognize only this single segment of

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the protein. Another strain (B10.D2 (H-2^d)) was examined, but these mice failed to recognize the protein (*12*). Thus, from two different strains examined, expressing between them 5 different MHC Class I genes, only a single CTL epitope was recognized. A similar paucity of CTL epitopes has been reported in other systems (*13*).

We have hypothesized that the variation observed in the CS protein may be selected by CTL (*14*). Since T cells only recognize antigenic epitopes in association with MHC molecules, such a selection would have to occur during a cellular stage in the life-cycle. We believe that CTL recognize and destroy hepatocytes infected with sporozoites expressing a CS protein epitope to which the CTL have previously been exposed. Hepatocytes containing sporozoites bearing variant CS proteins would escape destruction and thus be selected. Such variation, selected by CTL, would also affect the antibody response to the protein, as discussed below.

In murine systems, monoclonal antibodies directed against the CS protein repetitive epitope can passively protect an animal from live sporozoite challenge (*9*). This suggested that human vaccine development programmes should consider creating an immunogen capable of inducing a high-titre, high-affinity antibody response (*15–17*). Such an immunogen would consist of a B-cell epitope (the repetitive NANP epitope) conjugated to one or more T-cell epitopes as a source of T-cell help. T-cell epitopes could be of either parasite or non-parasite origin. However, for continual antibody-mediated protection, titres must remain high at all times. This is because there is no time following sporozoite inoculation and prior to penetration of hepatocytes for the antibody response to be boosted and to protect from the immediate sporozoite challenge. For a vaccine to be effective, titres would have to be high following vaccination, and would require natural boosting by the parasite itself. Antibodies would thus block sporozoites and the sporozoites would in turn boost antibody levels in readiness for the next sporozoite challenge. B cells derive help from T cells following uptake and processing of the antigen by the B cell. If the antigen (CS protein) contains a T-cell epitope against which the person has previously been immunized, there will be a secondary or boosted antibody response. If the immunogen contained T-cell epitopes from a protein other than the CS protein, such boosting would obviously not occur following sporozoite challenge. We initially examined the immunogenicity of the CS protein, in terms of the antibody response by different individuals, and then determined the helper T-cell epitopes on the protein.

At first we determined whether the antibody response to the protein was controlled by genes mapping within the MHC region. Different strains of H-2 congenic mice were immunized with either a *P. falciparum* CS-recombinant vaccinia virus (*18*) or with purified CS protein prepared from CS-recombinant baculovirus-infected cells (*11*). From nine different H-2 congenic strains examined, only two were high responders to the protein. A number of strains were either low responders or non-responders. It was possible to map the MHC genes responsible for a high response to I-A^b and I-A^k. To demonstrate that responsiveness was controlled by a 'responder' gene, rather than negatively controlled by a 'suppressor gene', the immunogenicity of the CS protein was determined for 'high responder × low responder' (F1) animals. Thus, B10.BR (H-2^k), B10.D2 (H-2^d), and B10.BR × B10.D2 (F1) were immunized and boosted with purified CS protein produced by CS-recombinant baculovirus-infected cells (Table 1). We observed that the B10.BR and the F1 animals were high responders, but that the B10.D2 animals were low responders. Thus, at least in these strain combinations, *immune suppressor* genes do not control the response to the CS protein.

Table 1: **Immune response genes within MHC control the humoral response to purified recombinant CS protein**[a]

Strain	Anti-$(NANP)_n$ titre	
	Primary	Secondary
B10.BR (H-2^k)		
1	256	4 096
2	512	> 16 384
3	512	> 16 384
4	64	256
5	512	> 16 384
B10.D2 (H-2^d)		
1	< 16	< 16
2	< 16	< 16
3	< 16	< 16
B10.BR × B10.D2 (F1)		
1	1024	> 16 384
2	64	4 096
3	64	4 096
4	1024	4 096
5	256	> 16 384

[a] Mice were immunized with 10 μg purified circumsporozoite protein (*P. falciparum*, 7G8 strain) (produced by recombinant baculovirus-infected cells, as described (*11*)) emulsified in CFA. Three weeks later, the animals were boosted with 10 μg aqueous CS protein. 'Primary' sera were taken just prior to the boost and 'secondary' sera were taken 10 days after the boost. Antibodies binding R32tet32 (*15*) were determined by ELISA as described (*18*). Titre is defined as the highest dilution of serum which produced an absorbance reading of at least 3 SD above the mean absorbance of a panel of normal mouse sera at the same dilution.

We (*19*) and others (*20*) had already shown that I-A^b-bearing animals recognized the repetitive epitope (NANP) as a T-cell epitope. However, no other strains responded to this epitope. Thus, there must have been another T-cell epitope present on the CS protein recognized by B10.BR mice. Using an algorithm to predict T-cell epitopes on the basis of helical amphipathicity, we located a major T-cell epitope in the carboxyterminal region of the protein (*18*). This epitope stimulated proliferating and helper T cells in B10.BR mice. It is also an immunodominant CD4 T-cell epitope for humans (*21*). To locate other possible CD4 T-cell epitopes, we used the overlapping set of peptides referred to above, which span the entire sequence. Both human and murine studies were performed (*11*, *21*). Both studies gave similar findings and demonstrated that for both species, most of the CD4 epitopes were located in the carboxyterminal, variant region of the protein. The human study, performed with lymphocytes from adults in the Gambia, also found that many adults (about 40%) were unable to respond to any of the overlapping set of peptides. This finding was analogous to the murine studies in two ways: firstly, many different H-2 congenic mouse strains were unable to respond to the CS protein, or were 'low responders' suggesting that there are few CD4 helper T-cell epitopes on the protein; secondly, similar regions of the protein were recognized by 'responders' from both species. This evidence supports the concept that physico-chemical properties of peptides are important in determining immunogenicity for T cells (*22*, *23*). According to the hypotheses, the structure of the processed peptide is important in determining whether it initially binds to the MHC molecule as a first step in stimulating T cells. Since MHC molecules from different species are similar in basic structure and share sequence homologies, a peptide that is immunogenic in one species may be expected to be immunogenic in another species. This would not imply that a minimal T-cell site in a mouse would be a minimal T-cell site in a human, merely that T-cell epitopes would come from similar or overlapping regions of the protein. The demonstration by this unbiased test that murine and human CD4 T-cell epitopes map to the same regions of the CS protein make the mouse a suitable candidate for mapping T-cell epitopes of vaccine interest for human pathogens. Although we have not performed family studies with humans from endemic areas, we believe that MHC genes also control the human response: firstly, humans and mice both recognize similar regions of the protein (*11*), suggesting that in humans, as in mice, MHC genes play an important role; secondly, we have preliminary evidence of association between certain DR types and response to the immunodominant peptides (*24*). However, by examining the proliferative response of non-immune individuals to peptides from the CS protein, Sinigaglia and colleagues have found that many different MHC DR antigens can present a slightly modified peptide from the constant region of the protein to a CS-specific T-cell clone (*25*). The discrepancy between these results and the restricted immune response observed in the Gambia need to be resolved.

Thus, we know that T cells play an important role in sporozoite immunity, both as effector cells (CTL) and as helpers for an antibody response; T-cell epitopes that stimulate CTL and helper T cells have been defined for the CS protein; but the epitopes occur in variant regions of the protein—an observation made more significant by the demonstration that the variant T-cell epitopes usually do not cross-react (*26*). How do we now use this information to help us design a vaccine? Firstly, we must realize that many other sporozoite-stage or liver-stage proteins may play important roles in a 'sporozoite' vaccine, and the immunogenicity and relevance of these proteins has scarcely been investigated. Secondly, the pattern of variation of the CS protein must be further examined. At present, the genes for six different CS proteins have been sequenced. The different strains represent laboratory isolates from different parts of the world. Whether such strains are at present occurring in endemic regions is not known. Neither is the degree of variation within given geographical regions known. The knowledge of such a pattern of variation will have major ramifications for vaccine design and may decide the feasibility of a CS-based vaccine. If variation within a given geographical region is limited, then the CS protein or proteins representative of that region may form a useful vaccine. From the perspective of an antibody-based vaccine, constant re-exposure to only a few different strains should boost the antibody response to the protein following vaccination. If, however, there was extensive variation within a locale, an individual may only rarely be exposed to vaccine strain sequences. Thus, even if he responded to the vaccine, his immune response would rarely be boosted. Although the pattern of variation is not known, we have recently observed that T lymphocytes from most individuals in an endemic region are capable of responding to at least one of six different peptides representing the immunodominant domains of three different CS proteins (*24*). This suggests that an antibody-oriented vaccine is definitely worth pursuing. The inclusion of a panel of immunodominant domains from different known CS sequences may prime T cells for exposure to various strains. However, there is an important difference between natural exposure followed by *in vitro* challenge (as in our experiment) and immunization followed by natural exposure. In the former case, lymphocytes exposed to parasites

over a lifetime may be recalled *in vitro*. Nothing is known about the history of strain exposure in any endemic region. In the latter case, the effectiveness of the vaccine will depend on the ability of lymphocytes primed by a (finite) panel of known variant peptides to respond to parasites *currently present* in the endemic region.

Knowledge of the pattern of variation would also have major ramifications on the design of a vaccine to stimulate cellular immunity. Any degree of variation of CS protein sequence within a given locale would almost preclude a role for CS-specific cytotoxic T lymphocyte immunity in vaccine design, unless the CTL epitopes on the different proteins (if indeed those particular CS proteins contained CTL epitopes) cross-reacted. Even then, such a vaccine would have a protective effect only on those individuals within the population whose CTL recognized those particular CTL epitopes. It would seem that the role of CTL in vaccine design could be augmented by the identification of CTL epitopes on many other sporozoite- or hepatic-stage proteins. Then it may be possible to use a large panel of different CTL epitopes that could stimulate a protective response in most people. However, it is sobering to realize that in endemic populations natural immunity to sporozoites may only rarely occur (*4*). This may indicate that a large number of protective CTL epitopes do not exist on the sporozoite- or the hepatic-stage forms. Other factors, however, may be responsible for the failure of natural sporozoite immunity to develop, including antigen dosage, malaria-induced immunosuppression, emergence of new antigenic strains in a given region, etc. Thus it is very important to continue to study the immune response to sporozoites, the geographical and dynamic pattern of variation, and to search for other important antigens which may play a role in protection.

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