Brief Definitive Report

Adjuvant-dependent Immune Response to Malarial Transmission-blocking Vaccine Candidate Antigens

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Summary

Immune responses in major histocompatibility complex (MHC)-disparate congenic mouse strains immunized with sexual stage malaria parasites or purified recombinant protein were adjuvant dependent. Whereas mice exhibited a limited antibody response to immunization with newly emerged *Plasmodium falciparum* gametes in Freund's adjuvant, all five congenic mouse strains responded to several transmission-blocking vaccine candidate antigens, when parasites were emulsified in a monophosphoryl lipid A (MPL) and trehalose dimycolate (TDM) adjuvant. The humoral response in those animals immunized with the antigen in a MPL/TDM adjuvant was helper T cell dependent, as evident by boosting of the antibody response after a second immunization. If the immunogen consisted of purified recombinant protein, then the immune response was not MHC class II limited in mice immunized with either complete Freund's adjuvant or TDM/MPL. The potential role of adjuvants in overcoming apparent immune nonresponsiveness and the implications for development of a malaria transmission-blocking vaccine are discussed.

Limited immunological recognition of vaccine candidate antigens may be a major obstacle in the development of a subunit vaccine against malaria. Limited humoral immune responses to the major candidate antigens from all stages of the parasite life cycle have been observed in MHC-disparate congenic animals as well as in human populations in malaria-endemic regions. These observations have included responses to: (*a*) the predominant sporozoite surface protein, the circumsporozoite protein (CSP) (1–5); (*b*) protective blood stage antigens, including the ring-infected erythrocyte surface antigen (RESA) (6, 7) and the major merozoite surface protein (MSP-1) (8); and (*c*) the sexual stage target antigens of transmission-blocking antibodies, Pfs230 and Pfs48/45 (9–12). These data are consistent with the hypothesis that host immune pressure has selected for parasites having a paucity of helper T cell epitopes within these molecules. Consequently, the malaria parasite now elicits a limited antibody response to these antigens (9, 11).

Good et al. (9) found that among six MHC-disparate congenic mouse strains immunized with *Plasmodium falciparum* gametes, only two strains recognized Pfs48/45 and two different strains recognized Pfs230. A single strain produced antibodies capable of immunoprecipitating a previously uncharacterized 40-kD surface radioiodinated gamete protein (9), Pfs40 (13). The poor immunogenicity of Pfs40 was similar to that of the other two known transmission-blocking immunity target surface antigens, Pfs230 and Pfs48/45, suggesting that Pfs40 might represent an additional target antigen of transmission-blocking antibodies (14, 15). The gene encoding Pfs40 was recently cloned, and recombinant Pfs40 protein (rPfs40) purified from bacteria (13). We evaluated the immune responses in five congenic mouse strains to native and rPfs40, and native Pfs230 and Pfs48/45, using a monophosphoryl lipid A (MPL) and trehalose dimycolate (TDM) adjuvant in parallel with CFA and IFA. The results of these studies challenge the current notion of why there is a limited immune response to malarial vaccine candidate antigens.

Materials and Methods

Parasites. Mature *P. falciparum* gametocytes of clone 3D7 (16) were obtained by in vitro culture as previously described (17). After induction of gametogenesis and exflagellation (18), gametes/zygotes were purified using a discontinuous Nycodenz gradient (19) and frozen in aliquots at −70°C.

Recombinant Protein Production. Recombinant Pfs40 (amino acids 27–374, i.e., without the putative secretory signal sequence) was expressed in the prokaryotic expression vector pIH902 (a gift from Dr. P. Riggs, New England Biolabs, Beverly, MA) as a fusion protein with maltose binding protein (MBP), purified, and cleaved with factor Xa as previously described (13).

Immunizations. Five MHC-disparate congenic mouse strains of the B10 genetic background (kindly provided by Dr. R. Schwartz, LCMI, NIAID, Bethesda, MD) were used for immunization studies. One group of animals received an intraperitoneal injection of 100 μg of factor Xa–cleaved rPfs40 (or MBP) emulsified in CFA, and were boosted with antigen in IFA 21 d after the primary injection. Immune sera were collected 31 d after the primary immunization.

A second group of congenic animals was immunized with a primary intraperitoneal injection of 5×10^6 *P. falciparum*–purified

newly emerged gametes/zygotes in PBS emulsified in either CFA/IFA or in a final volume of 200 μl PBS containing 50 μg each of MPL and TDM, 4 μl squalene oil, and 0.02% Tween-80 (MPL/TDM adjuvant, R-700; Ribi Immunochem, Hamilton, MT). These animals received two subsequent injections of 5×10^6 gametes/zygotes in IFA or MPL/TDM adjuvant, respectively, at 21 and 31 d after the primary injection. Control animals received primary and subsequent injections of PBS alone in CFA, or MPL/TDM. Immune sera were collected 28 and 41 d after the primary immunization.

An additional group of congenic animals received a primary intraperitoneal injection of 5×10^6 *P. falciparum* gametes in MPL/TDM adjuvant and an identical injection after 10 wk. To evaluate boosting of the immune response to antigen emulsified in MPL/TDM adjuvant, immune sera were collected 7 d after the primary, and 1 d before and 7 d after the secondary immunization.

Immunological Studies. Surface radioiodination of live parasites and immunoprecipitation and SDS-PAGE of SDS/Triton X-100–solubilized gametes of *P. falciparum* were performed as described (9). Rabbit 129 sera and Western blot analysis of rPfs40 (13) and mAb IIC5B10 (20) were generated and used as previously described.

Results and Discussion

Helper T Cell Epitopes in Recombinant Pfs40 Are Recognized by All Congenic Mouse Strains. The immune response to Pfs40 in six congenic mouse strains immunized with unfractionated total *P. falciparum* gamete antigens emulsified in CFA was limited to one strain expressing a single MHC class II allele (9). Production of purified rPfs40 has now allowed us to specifically evaluate the immune response to this protein in the absence of other parasite molecules that might influence immunogenicity of Pfs40. rPfs40, expressed as MBP-Pfs40 fusion protein without the putative signal peptide, was purified after factor X_a cleavage. Five MHC-disparate congenic mouse strains were immunized with rPfs40 emulsified in CFA and boosted once with rPfs40 in IFA. Surprisingly, all five strains produced antibodies recognizing the recombinant protein by immunoblot (Fig. 1). Upon subsequent immunization, all five strains showed a boost in antibody response by immunoblot (data not shown), indicating that the immune response was helper T cell dependent rather than T cell independent. Sera from mice immunized with MBP alone immunoblotted only the MBP portion of the fusion protein (Fig. 1).

The Limited Immune Response to Native Pfs40 Is Adjuvant Dependent. The MHC class II–independent immune response to rPfs40 in congenic mice is in sharp contrast to the MHC class II–dependent response to the native Pfs40 presented in the complex mixture of antigens present in whole gametes. Incomplete cleavage of the fusion protein and subsequent contribution of helper T cell epitopes from the MBP portion of the fusion protein would be a trivial explanation for this difference. This explanation is unlikely, however, because there was no evidence of uncleaved fusion protein by immunoblotting or protein staining (data not shown). An alternate explanation is that an immunogen of whole gametes contains a mixture of gamete proteins, carbohydrates, lipids, and membrane structures, one or some of which limit the immune response to Pfs40 when presented in a specific adjuvant. To examine this possibility, we evaluated the effect of adjuvant on MHC control of antibody response and antigen specificity in congenic mice immunized with whole gametes.

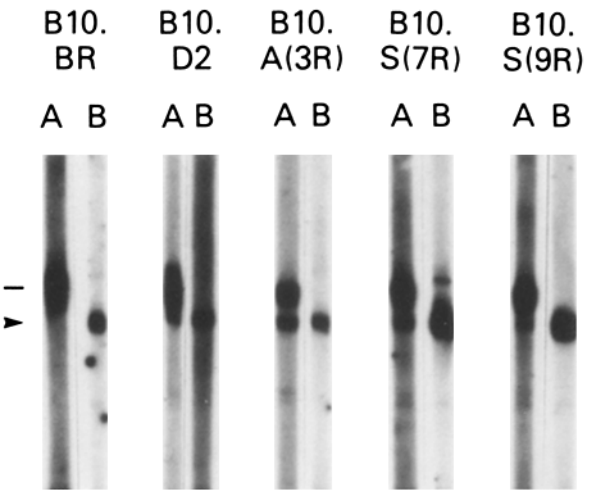

Figure 1. Immunogenicity of recombinant Pfs40 emulsified in Freund's adjuvant and injected into five congenic mouse strains. Immune sera from congenic mouse strains (B10.BR, B10.D2, B10.A[3], B10.S[7R], B10.S[9R]) immunized with either affinity-purified maltose binding protein (MBP; lanes *A*) or with rPfs40 partially purified from factor X_a–cleaved, affinity-purified MBP-rPfs40 fusion protein (lanes *B*) were assayed by immunoblot analysis of factor X_a–cleaved, affinity-purified MBP-rPfs40 fusion protein size fractionated by SDS-PAGE under nonreducing conditions and electroblotted to nitrocellulose. Although no intact MBP-rPfs40 fusion protein was detectable by Western blot, the partially purified preparations of rPfs40 contained some MBP, and elicited anti-MBP antibodies in some mouse strains (e.g., B10.A[3R]). The solid line indicates the location of rPfs40 on the nitrocellulose blot, and the arrowhead indicates the location of MBP.

MPL, a nontoxic LPS derivative of Gram-negative bacterial endotoxin (21), and trehalose dimycolate, a component of mycobacterial cell walls (22), have been shown to increase antibody response to a variety of antigens, including malarial proteins (23, 24). The combination of MPL and TDM is a particularly potent immunological adjuvant (25, 26). Therefore, the immune response in congenic mice immunized with *P. falciparum* gametes emulsified in MPL/TDM was evaluated in parallel with that elicited by gametes emulsified in CFA. The response using CFA was dramatically limited (Fig. 2 *A*). This limited response to immunization with newly emerged, untransformed *P. falciparum* gametes, emulsified in CFA, was similar but not identical to the results noted by Good et al. (9), using 5-h-old zygotes. The inconsistencies between our findings and those of Good et al. (9) probably represent minor differences in experimental design. In marked contrast to the mice immunized with gametes in CFA, when immunized with gametes in MPL/TDM, all five congenic mouse strains, independent of MHC class II alleles expressed, developed antibodies capable of immunoprecipitating Pfs40 and Pfs48/45 (Figs. 2 *A* and 3). Only a single strain, B10.S(9R), immunoprecipitated Pfs230 (Fig. 3).

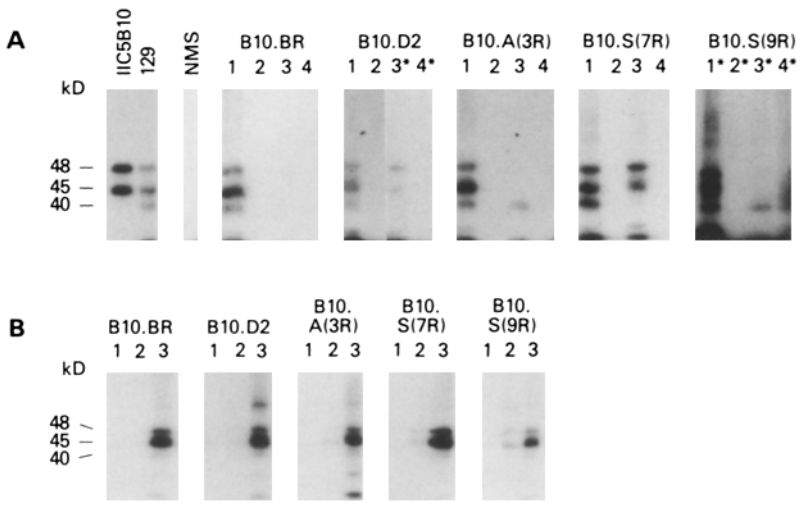

Figure 2. (*A*) Immunoprecipitation of ^{125}I-labeled Pfs48/45 and pfs40 surface proteins by preimmune (lanes *2* and *4*) or immune sera (lanes *1* and *3*) from five congenic mouse strains immunized with gametes and zygotes emulsified in MPL/TDM (lanes *1* and *2*) or in CFA (lanes *3* and *4*), using methods described in Fig. 1. Also shown are immunoprecipitations with mAb recognizing Pfs48/45 (*IIC5B10*), rabbit polyclonal sera (*129*) (reference 34) recognizing Pfs48/45 and Pfs40, and normal mouse sera (*NMS*). * Autoradiograph is from a 3-d exposure rather than overnight. (*B*) Immunoprecipitation of ^{125}I-labeled Pfs48/45 and Pfs40 surface proteins by immune sera 7 d after a primary immunization (lanes *1*), or 1 d before (lanes *2*) or 7 d after (lane *3*) a secondary immunization with gametes and zygotes emulsified in MPL/TDM.

Immunization with Gametes in MPL/TDM Elicits T Cell–dependent Antibody Responses. Noncognate (T cell–independent) help, perhaps mediated by IL-2, is one effect an adjuvant may have on the immune response to overcome genetic nonresponsiveness to an immunogen (for example, see references 27 and 28). Such a T cell–independent immune response would not be expected to elicit a secondary immune response on subsequent immunizations. Therefore, to evaluate whether subsequent immunizations elicit a secondary antibody response to these gamete proteins, sera were collected after primary and secondary immunizations of additional animals of all five congenic mouse strains immunized with *P. falciparum* gametes in MPL/TDM adjuvant. Immunoprecipitations with these sera clearly demonstrated a boost of antibody response to Pfs40 and Pfs48/45 in all of the congenic mouse strains (Fig. 2 *B*). Little or no detectable antibody response to Pfs40 and Pfs48/45 was present in primary immune sera or in sera immediately before the secondary immunization. Both proteins were immunoprecipitated, however, by sera obtained 7 d after the secondary immunization given 10 wk later. Nonspecific polyclonal B cell stimulation by MPL/TDM adjuvant is unlikely to explain this universal response. Rather, these data suggest that the antibody response elicited by *P. falciparum* gametes in the adjuvant is T cell dependent.

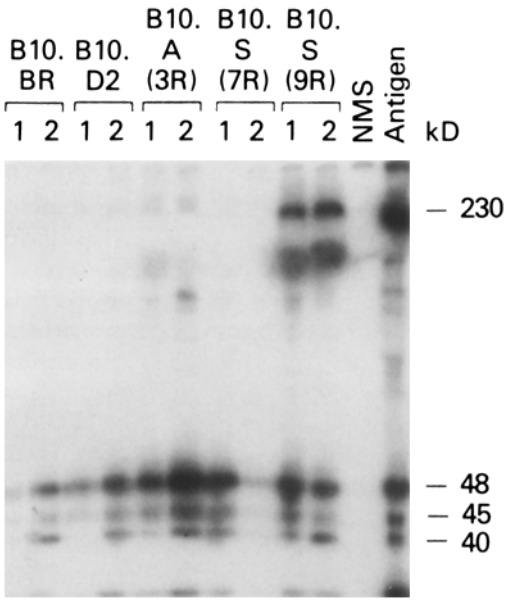

Figure 3. Immunoprecipitation of *P. falciparum* ^{125}I-labeled surface proteins by immune sera from congenic mouse strains immunized with gametes and zygotes emulsified in MPL/TDM. Two animals of each of five congenic mouse strains (B10.BR, B10.D2, B10.A[3], B10.S[7R], B10.S[9R]) were immunized and boosted with *P. falciparum* gametes and zygotes using TDM/MPL adjuvant. Serum from each of these individual animals (lanes *1* and *2*) and from an animal immunized with adjuvant alone (*NMS*) were used in immunoprecipitations of SDS/Triton X-100 extracts of surface radioiodinated *P. falciparum* gametes and zygotes. Bound ^{125}I-labeled surface proteins and total ^{125}I-labeled extract (*Antigen*) were size-fractionated by SDS-PAGE. An autoradiograph of the dried gel shown is from an overnight exposure. Location of target proteins Pfs230 (230), Pfs48/45 (48, 45), and Pfs40 (40) are indicated.

Implications for Vaccine Development. These immunogenicity studies produced two unexpected results. First, the immunorestricted antibody response in MHC-disparate congenic animals, immunized with *P. falciparum* gametes, was adjuvant dependent and not necessarily due to an absence of helper T cell epitopes. When CFA was used, we confirmed the apparent MHC class II–associated nonresponsiveness to the surface proteins, Pfs40 and Pfs48/45. In contrast, though, to previous studies, we observed an MHC class II–independent but T cell–dependent immune response to the same proteins using MPL/TDM adjuvant. Second, unlike the response to Pfs40 in the milieu of whole gametes emulsified in CFA, the antibody response to purified, recombinant Pfs40 in CFA was not class II MHC dependent.

The divergent immune responses to whole gametes emulsified in different adjuvants may result from alterations in antigen processing/presentation and/or elimination of immune suppression. For instance, TDM/MPL adjuvant may allow the presentation of additional helper T cell epitopes by macrophages and other APC, mediated through changes in antigen processing or presentation pathways that do not occur with CFA. Both MPL (25) and TDM (29) activate macrophages and stimulate cytokine production. This acti-

vation may fundamentally alter antigen processing. Additionally, chemical and conformational changes that may occur when an antigen is emulsified in different adjuvants may alter specific sites of antigen proteolytic processing and, therefore, alter antigen presentation.

Alternatively, it is possible that the MPL/TDM adjuvant interferes with specific suppressor T cell proliferation, allowing expression of previously suppressed T cell–dependent antibody responses. For example, MPL specifically inactivates suppressor T cells but appears to have little effect on helper T cell activity (30). Notably, MPL overcomes low-dose immunological paralysis and potentiates the immune response to type III pneumococcal polysaccharide by eliminating inhibitory effects of suppressor T cells (31). In the case of *P. falciparum* sexual stage antigens, any adjuvant-dependent immunosuppressive model must be antigen specific to account for adjuvant-independent widespread immunogenicity of two other sexual stage proteins, Pfs25 and Pfg27/25, in congenic mouse strains (9) and in humans (11), respectively. Furthermore, the MHC class II–independent immune response to rPfs40 alone, and the contrasting limited response to the native molecule in immunization studies with whole gametes (using identical adjuvant systems), suggests that a suppressor determinant(s), if involved, is in an unrelated molecule(s). Therefore, unlike CFA, MPL/TDM adjuvant may be capable of overcoming an antigen-specific suppressor response in animals immunized with total gametes that remains present when CFA is used. A similar situation, that is, the presence of a suppressor determinant in a molecule distinct from the antigen of interest, seems to exist in mice infected with the nematode *Ascaris suum*. A limited antibody response to the nematode 14-kD excretory/secretory antigen, after natural infection, can be overcome by immunization with purified protein in CFA (32).

The data presented here, therefore, suggest that recognition of T cell epitopes in at least two gamete surface antigens, Pfs48/45 and Pfs40, is not MHC restricted as previously indicated, or that genetic nonresponsiveness can be overcome at the level of antigen processing and/or by elimination of specific T cell suppression. This interpretation is consistent with data from field studies. Although sera from humans living in malaria-endemic regions have limited recognition of transmission-blocking gamete surface proteins (10–12), there appears to be no association between the immune responsiveness in individuals and their MHC class II haplotypes. Individuals homozygous for either of the two most common HLA-DR alleles responded to each of the candidate molecules (33). In another recent study, the MHC-restricted response to a malaria parasite blood stage protein, MSP-1, could be overcome in at least one congenic mouse strain by immunization with antigen in a MPL-derivative adjuvant (8). These results and those reported here, while still preliminary, challenge the current pessimism that limited T cell epitopes in critical vaccine candidate antigens may make a subunit malarial vaccine infeasible, and suggest a more optimistic outlook for subunit malarial vaccines.

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