Inhibition of 19-kDa C-Terminal Region of Merozoite Surface Protein-1-Specific Antibody Responses in Neonatal Pups by Maternally Derived 19-kDa C-Terminal Region of Merozoite Surface Protein-1-Specific Antibodies but Not Whole Parasite-Specific Antibodies

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Inhibition of 19-kDa C-Terminal Region of Merozoite Surface Protein-1-Specific Antibody Responses in Neonatal Pups by Maternally Derived 19-kDa C-Terminal Region of Merozoite Surface Protein-1-Specific Antibodies but Not Whole Parasite-Specific Antibodies

Danielle I. Stanisic,*† Laura B. Martin,2*† Michelle L. Gatton,2*† and Michael F. Good3*†

Immunizing pregnant women with a malaria vaccine is one approach to protecting the mother and her offspring from malaria infection. However, specific maternal Abs generated in response to vaccination and transferred to the fetus may interfere with the infant's ability to respond to the same vaccine. Using a murine model of malaria, we examined the effect of maternal 19-kDa C-terminal region of merozoite surface protein-1 (MSP119) and Plasmodium yoelii Abs on the pups’ ability to respond to immunization with MSP119. Maternal MSP119-specific Abs but not P. yoelii-specific Abs inhibited Ab production following MSP119 immunization in 2-wk-old pups. This inhibition was correlated with the amount of maternal MSP119 Ab present in the pup at the time of immunization and was due to fewer specific B cells. Passively acquired Ab most likely inhibited the development of an Ab response by blocking access to critical B cell epitopes. If a neonate’s ability to respond to MSP119 vaccination depends on the level of maternal Abs present at the time of vaccination, it may be necessary to delay immunization until Abs specific for the vaccinating Ag have decreased. The Journal of Immunology, 2004, 172: 5570–5581.
the critical B cell epitopes within the vaccine, preventing the binding of the infant’s B cells to the Ag, with the level of masking dependent on the ratio of maternally derived Abs to vaccine Ag. The maternal Ab-Ag complexes are then taken up by APCs, processed, and presented, allowing for T cell priming in the absence of a B cell response.

It is unknown whether this hypothesis can be applied to all infant immunizations in the presence of maternally derived Abs. Previous studies undertaken in rodents have aimed to examine the effect of maternally derived malaria parasite-specific Abs on the vaccination of infant pups. It was observed that maternally derived \textit{Plasmodium yoelii}-specific Abs generated in response to vaccination and transferred by lactation interfered with the pups’ ability to generate a protective immune response to vaccination with the whole parasite (9). Additionally, there was both suppression of T cell priming by the vaccine and generation of specific T helper cells involved in Ab production. This inhibition was overcome by administration of a second dose of the vaccine, 10 or more days after the first (10).

A study by Sedegah et al. (11) examined the effect of maternal immune status on the induction of a protective CD8 T cell-dependent response against malaria in mouse pups using a heterologous DNA prime, viral boost strategy. They observed that maternally derived Abs could interfere with the ability of neonatal mice to produce Abs whereas CD8 T cell responses were unaffected. Because the vaccine they were testing (\textit{P. yoelii} circumsporozoite protein) relies primarily on the induction of a CD8 T cell response for protection, the effect of maternal Abs on the pup’s ability to produce Abs is not a great concern for this particular vaccine strategy. It would be problematic for a vaccine candidate such as the 19-kDa C-terminal region of merozoite surface protein-1 (MSP1\textsubscript{19}),\textsuperscript{4} which requires the production of high titer Abs for a protective immune response.

Infants in their first few months of life appear to be somewhat resistant to the severe manifestations associated with malaria infection (12–15), with some attributing this protection to the presence of maternally derived parasite-specific Abs (16–18). After this time, the levels of maternally derived Abs in the infant decrease, whereas parasite densities and duration of infections increase. Studies examining the effect of maternally derived Abs on time to patent parasitemia or onset of clinical disease have produced conflicting results, with some studies finding an association between total parasite-specific Ab and parasitemia in the infant (19) and others finding no association at all (20–22). Interestingly, a few studies have observed a relationship between the level of Ab at birth specific for MSP1\textsubscript{19} (4) and resistance to clinical disease in infants (22, 23). Given that the natural Ab response to MSP1\textsubscript{19} in malaria endemic regions has been observed to increase with age (24) and is correlated with protection in older children (25, 26), the potential role of maternally derived MSP1\textsubscript{19}-specific Abs in the infant cannot be underestimated. Indeed, immunizing pregnant women with MSP1\textsubscript{19} may be one approach to protecting infants until they develop their own protective immune response.

Recent human malaria clinical trials using the peptide vaccine SPf66 (27–29) have included infants as their study subjects. These studies have primarily examined the infants' response to the vaccine and any protection from clinical disease. Although the infants were able to produce an Ab response following vaccination, the levels of SPf66-specific Abs fell rapidly after the final dose (13).

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\textsuperscript{4} Abbreviations used in this paper: HIS, hyperimmune serum; MSP1\textsubscript{19}, 19-kDa region of merozoite surface protein-1; yMSP1\textsubscript{19}, recombinant MSP1\textsubscript{19} corresponding to \textit{P. yoelii} protein produced with a C-terminal 6-histidine in \textit{Saccharomyces cerevisiae}; NMS, normal mouse serum; PPD, purified protein derivatives.

A number of possible mechanisms were postulated for the lack of a sustained Ab response in these infants, including mopping up by maternally derived Abs of the inoculum preventing a functional response to the vaccine. The only Abs (maternally derived or of infant origin) specific for the Ag that these infants would have before vaccination would be a result of malaria infection. It is yet to be determined whether infants born to mothers immunized with a malaria vaccine candidate can themselves be immunized with the same vaccine. The maternally derived Abs generated against the vaccine would be the result of a more focused immune response with greater potential to interfere with the infant’s response to the same vaccine.

We recently examined the effect of maternally derived Abs against MSP1\textsubscript{19} and \textit{P. yoelii} on the pups’ ability to respond to malaria infection (30). It was shown that although the maternally derived Abs suppressed the growth of the parasite in the pups, they also interfered with the development of the pups’ own Ab response to the parasite by altering the fine specificity of the response. Because specific maternally derived Abs direct the pups’ Ab response toward an alternate epitope after malaria infection, it is conceivable that these maternal Abs may also interfere with the development of the pups’ immune response to immunization. Therefore, it was decided to investigate the effect of MSP1\textsubscript{19} and \textit{P. yoelii}-specific maternally derived Abs on the ability of infant pups of various ages to respond to MSP1\textsubscript{19} immunization. Both Ab and T cell responses of the pup were examined. It was found that high titers of maternally derived MSP1\textsubscript{19}, but not \textit{P. yoelii}-specific Abs, could interfere with the production of an MSP1\textsubscript{19}-specific Ab response in pups following vaccination. Studies in adult mice suggested that the presence of specific Ab affected the number of B cells producing MSP1\textsubscript{19}-specific Abs in response to MSP1\textsubscript{19} vaccination. Although proliferative T cell responses were unaffected in the presence of specific Ab, we cannot exclude the possibility that maternal T cells were contributing to the proliferation of T cells from pups suckled on an MSP1\textsubscript{19}-immune mother. These results infer that MSP1\textsubscript{19} immunization of infants born to MSP1\textsubscript{19}-immunized mothers may need to be delayed until the level of maternally derived Abs has decreased.

## Materials and Methods

### Mice and parasites

Female BALB/c (H-2\textsuperscript{b}) and C57BL/6 (H-2\textsuperscript{b}) mice were obtained from the Animal Resources Centre, Willetton, Australia. Mice were housed in the Queensland Institute of Medical Research animal facility under specific pathogen-free conditions. All experiments were performed in compliance with the Queensland Institute of Medical Research Animal Ethics Committee requirements.

The malaria parasite used was \textit{P. yoelii} YM. The parasites were maintained by i.p. passaging of 10\textsuperscript{6} parasitized erythrocytes into recipient mice. The parasites, as infected RBC, were cryopreserved in liquid nitrogen.

### Recombinant protein

Recombinant MSP1\textsubscript{19} corresponding to \textit{P. yoelii} protein was produced with a C-terminal 6-histidine in \textit{Saccharomyces cerevisiae} (yMSP1\textsubscript{19}) (31). It was provided as a gift by Dr. A. Stowers (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

### Immunization of mice for MSP1\textsubscript{19}-specific Ab production

Female mice were immunized s.c. with 20 μg of MSP1\textsubscript{19} in CFA. Equal amounts (10 μg at each site) were delivered to the tail base and abdomen. The mice were boosted with 20 μg of MSP1\textsubscript{19} in IFA s.c. on day 21 at the base of the neck, then i.p. on days 42 and 56. They were given a final boost i.p. on day 63 with the same amount of MSP1\textsubscript{19} in PBS. On completion of the immunization schedule, blood was collected from the mice every week. The sera from the individual mice were pooled, and the Ab titer was determined by ELISA. The pooled hyperimmune sera (HIS) were used in passive transfer studies.
Infection of mice for P. yoelii-specific Ab production

Mice were infected i.p. with 1 × 10^7 P. yoelii YM parasitized erythrocytes. When the parasitaemia had reached ~40%, the mice were treated on 3 consecutive days with 0.2 ml of 1 mg/ml pyrimethamine dissolved in PBS-Tween 80. This infection and drug cure regimen was repeated an additional four times with 3 wk between the last day of drug treatment and the new cycle of infection and drug cure. Three weeks after the completion of the infection and drug cure regimen, blood was collected from the mice weeded to the sera from the individual mice were pooled, and the Ab titer was determined by ELISA. The pooled HIS were used in passive transfer studies.

Immunoassay and infection of female mice before breeding

Female mice were immunized according to the schedule outlined for generation of yMSPI19-specific Ab with the following changes: the day 42 boost with MSP119 in IFA was given s.c. and the day 56 boost with MSP119 was given i.p. in PBS. This was followed by a boost with MSP119 in PBS on day 63. A separate group of female mice also underwent infection and drug cure according to the schedule outlined for generation of P. yoelii-specific Ab.

Immunoassay of mice for passive transfer study

Groups of five adult C57BL/6 mice were given 0.5-ml i.p. injections of BALB/c MSP119 HIS, P. yoelii HIS, or normal mouse serum (NMS) on days −2, −1, 0 and 1. Serum was produced according to the methods outlined above.

Immunoassay of mice with MSP119

Pups were immunized s.c. with 20 μg of MSP119 in CFA. Equal amounts (10 μg at each site) were delivered to the tail base and abdomen. The mice were boosted s.c. on day 21 at the base of the neck with the same amount of Ag in IFA.

Breeding of mice

Female mice were hormone treated to facilitate timed breeding experiments. They were given 100 μl of a 20-IU/ml solution of serum gonadotropin (Folligon; Intervet, Bendigo, Australia) followed 46–48 h later by 100 μl of a 20-IU/ml solution of chorionic gonadotropin (Chorulon; Intervet). Hormone-treated female mice were housed with male mice. From 19 to 21 days later, female mice were monitored for delivery of pups.

Ab assay

Serum Ab levels were assayed by ELISA; 96-well plates were coated with 0.5 μg/ml MSP119 in coating buffer and incubated overnight. The wells were then blocked with 1% BSA-PBS for 1 h at 37°C. After washing with 0.05% Tween 20-PBS, serum previously diluted 1/10 in PBS was added to the wells, serially diluted, and incubated for 1 h at 37°C. The plates were washed again, and a goat anti-mouse IgG HRP conjugate (The Binding Site, Birmingham, U.K.) diluted 1/3000 in 1% BSA-PBS was added and incubated for 1 h at 37°C. For IgM detection, a goat anti-mouse IgM HRP conjugate (The Binding Site) was diluted 1/6000 in 1% BSA-PBS. After further washing, substrate solution (ABTS; Sigma-Aldrich, St. Louis, MO) was added. After a 30-min incubation at room temperature, the OD was determined at 405 nm on a Tecan Spectra plate reader (Tecan Instruments, Salzburg, Austria).

For the ELISAs using allotype-specific reagents, the above method was used with the following changes: the goat anti-mouse IgG2a or IgG2a Abs (BD PharMingen, San Jose, CA) were added at a concentration of 0.2 μg/ml after washing serum from the plate and then incubated at 37°C for 45 min. After washing the streptavidin-peroxidase HRP conjugate (BioSource International, Camarillo, CA) was added at a concentration of 0.1 μg/ml and incubated at room temperature for 45 min. After washing, the substrate was added and the plate was read according to the method outlined above.

For the inhibition ELISAs, coating, blocking, and addition of diluted sera were the same as described above. However, an extra step involving the incubation of mAbAb32 (1 μg/ml) for 1 h was performed after the serum had been washed from the plate. The mAbAb32 was then washed from the plates before the addition of a 1/3000 dilution of a goat anti-mouse IgG3 HRP conjugate (The Binding Site) to detect the level of mAbAb32 binding to MSP119.

ELISPOT for detection of Ab-secreting B cells

Multiscreen assay plates (Millipore, Bedford, MA) were coated with 0.5 μg/ml MSP119, a carbonate buffer overnight at 4°C. After the wells were emptied, they were blocked for 2 h at 37°C with 5% FCS. Following three washes in 0.05% Tween 20-PBS, the wells were filled with medium and incubated at 37°C for 30 min. This washing step was repeated. After a wash in PBS, 200 μl of spleen or lymph node cell suspensions were added at a concentration of 2 × 10^6 cells/ml. The plates were incubated for 5 h at 37°C with 5% CO2 in a humid atmosphere. The cell suspensions were removed from the plates and then washed with PBS three times. A 1/5000 dilution of biotinylated goat anti-mouse IgG2a Ab (BD PharMingen) in 5% FCS-PBS was added to the wells and incubated overnight at 4°C. After three washes in PBS, a 1/500 dilution of streptavidin–alkaline phosphatase (BD PharMingen) in 5% FCS-PBS was added to the wells and incubated at 37°C for 2 h. After a wash in PBS, substrate solution (5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium-alkaline phosphatase; Sigma-Aldrich) was added and incubated in the dark at room temperature for 15 min. The reaction was stopped with water. Plates were read using a stereomicroscope.

Lymphocyte proliferation assay

Mice were immunized in the hind footpads with 50 μg of MSP119 emulsified in CFA. From 7 to 9 days later, the draining popliteal and inguinal lymph nodes were removed. Cells were suspended at 2.5 × 10^6 cells/ml in EMEM (Trace Biosciences, Castle Hill, Australia) containing 2-ME at a final concentration of 5.5 × 10^-3 M and 2% heat-inactivated NMS; 200 μl of this cell suspension were added to 96-well plates. Cells were cultured in triplicate with varying concentrations of Ags (30 and 10 μg/ml MSP119 and purified purified protein derivative (PPD)) and a mitogen (10 μg/ml Con A) for 72 h at 37°C and 5% CO2. The plates were pulsed with [3H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ), and incorporation of radiolabel was measured 18–24 h later by β emission spectroscopy.

Cell staining with PKH26 for measurement of T cell proliferation

Mice were immunized with 50 μg of MSP119 emulsified in CFA in the hind footpads. From 7 to 9 days later, the draining popliteal and inguinal lymph nodes were removed, and cell suspensions were prepared. The cells were pelleted and resuspended in Diluent C (Sigma-Aldrich) at a concentration of 2 × 10^6 cells per ml of diluent. Following this, a 3 μM PKH26 dye solution was made by diluting 1 M PKH26 dye stock (Sigma-Aldrich) in Diluent C. Dye solution was added to the suspended cells at 1 ml of 3 μM dye solution per 2 × 10^7 cells. After a 2-min incubation, double the volume of PBS was added to the cells to stop dye incorporation and incubated for a further minute. The labeled cells were washed in phenol red-free IMDM twice before adding them to round-bottom 96-well plates at a concentration of 2.5 × 10^6 cells/ml. Cells were cultured in duplicate in phenol red-free medium with varying concentrations of Ag or mitogens for 96 h at 37°C, 5% CO2.

Cell staining with Abs for flow-assisted cytometry

After the 96-h incubation for the PKH26-stained cells, the cells were pelleted. FITC-labeled anti-mouse CD3 (BD PharMingen) and biotin-labeled anti-mouse H-2d (BD PharMingen) Abs were added to the cells diluted in FACs buffer (1% BSA-PBS) each at a concentration of 1/50 in a staining volume of 50 μl. After a 1-h incubation, the cells were washed three times with FACs buffer and streptavidin–quantum red (Sigma-Aldrich) was added to the cells at a concentration of 1/7 also in a staining volume of 50 μl. The cells were incubated for a further hour; this was followed by three washes in FACs buffer. The cells were then pelleted and immediately resuspended in ice-cold FACs fixative (2% paraformaldehyde, 2% BSA-PBS), ensuring that all samples were resuspended in the same final volume.

Analysis of cell proliferation and cell phenotype

The flow cytometer (FACSCalibur; BD Biosciences, San Diego, CA) was set to acquire events for 80 s. The flow acquisition collection allowed the relative number of viable cells in each sample to be determined, directly measuring the magnitude of the proliferative cellular response. After initially gating on the cell population of interest (as determined by cell viability), it was necessary to define the proliferating population of cells. The intensity of the PKH26 staining decreases as the cells proliferate, so that on a graph representing fluorescence intensity, the proliferating population would shift left. To define the proliferating population of cells, the profile of cells that had not been incubated with Ag was compared with cells that had been incubated with a mitogen such as Con A or PPD. Between the main population of cells with high fluorescence intensity for PKH26 and cells that appeared not to stain with PKH26 (lowest fluorescence intensity) was a region with few cells in the medium control and many cells in the samples incubated with Con A, PPD, or MSP119.
The cells in this region were deemed to be the proliferating population, and CD3 and H-2\textsuperscript{d} staining were examined for these cells to determine their phenotype. Data was analyzed using the CellQuest software (BD Biosciences).

Statistical analysis

Experiments were analyzed using the Mann-Whitney \( U \) test and Spearman rank correlation. The total amount of Ab produced within the study period was estimated as the area under the Ab curve. Differences in total Ab produced between the groups were investigated using the Mann-Whitney \( U \) test. Correlations between the amount of Ab present at the time of immunization and total amount of Ab produced within the study period were tested using the Spearman’s rank correlation. The \( t \) test was used to compare the level of H-2\textsuperscript{d} staining on cells from pups with the level of H-2\textsuperscript{d} staining on cells from parental mouse strains. Statistical analysis was completed using results from individual mice.

Results

Effect of passively transferred \( P. yoelii \) and MSP1\textsubscript{19} Abs on the Ab response following immunization

To determine the effect of \( P. yoelii \) and MSP1\textsubscript{19}-specific Abs on the development of the Ab response following MSP1\textsubscript{19} immunization, we exploited differences in murine strain allotype genetics that allowed differentiation between passively administered Abs and Abs produced by recipient mice (32). BALB/c (Igh-1a) HIS containing IgG2a\textsuperscript{b} Abs was transferred into C57BL/6 mice (Igh-1b), which would produce IgG2a\textsuperscript{b}, but not IgG2a\textsuperscript{c}, in response to immunization. To ensure that any effect seen was directly related to the presence of Abs, and not a function of the less mature immune system in an infant mouse, this experiment was undertaken in adult mice.

Groups of five female C57BL/6 mice were given i.p. injections of BALB/c MSP1\textsubscript{19} HIS, \( P. yoelii \) HIS, or NMS on days –2, –1, 0, and 1 relative to the day of immunization. The MSP1\textsubscript{19} and \( P. yoelii \) HIS were generated using protocols known to induce high titer Abs capable of protecting mice from parasite challenge (see Materials and Methods for production of HIS). They were then immunized on day 0 with 20 \( \mu \)g of MSP1\textsubscript{19} in CFA delivered equally to the tail base and abdomen.

The mice that received NMS had no detectable MSP1\textsubscript{19}-specific Abs at the time of immunization (Fig. 1A). Total MSP1\textsubscript{19}-specific IgG (closed symbols) present in the mice at the time of immunization was greater in the mice that received MSP1\textsubscript{19}-specific HIS than in the mice that received \( P. yoelii \)-specific HIS (Fig. 1, B and C). The MSP1\textsubscript{19}-specific Ab titers in these two groups of mice on day 0 reflect the normal reactivities of \( P. yoelii \)- and MSP1\textsubscript{19}-specific HIS to MSP1\textsubscript{19}.

All of the mice, irrespective of the specificity of the transferred serum, had detectable MSP1\textsubscript{19} IgG2a\textsuperscript{b} 7 days after immunization (Fig. 1). However, there were differences between the groups in the level of MSP1\textsubscript{19} IgG2a\textsuperscript{b} (open symbols) produced in response to MSP1\textsubscript{19} immunization. The mice that received NMS had the highest titers of MSP1\textsubscript{19}-specific IgG2a\textsuperscript{b} 21 days after immunization with MSP1\textsubscript{19} (Fig. 1A, open symbols) in contrast to the mice that received MSP1\textsubscript{19} HIS, who had the lowest titers of MSP1\textsubscript{19} IgG2a\textsuperscript{b} (Fig. 1C).

The relationship between the total MSP1\textsubscript{19}-specific IgG present on the day of immunization and the total amount of MSP1\textsubscript{19}-specific IgG2a\textsuperscript{b} produced in the first 20 days (see Materials and Methods) for each individual mouse was examined. For all mice, there was a significant inverse correlation between the total amount of MSP1\textsubscript{19}-specific Ab present on the day of immunization and the total amount of MSP1\textsubscript{19}-specific IgG2a\textsuperscript{b} produced in response to MSP1\textsubscript{19} immunization (\( r = -0.761, p = 0.004 \)).
All pups born to immune mothers had MSP1\textsubscript{19}-specific IgG in their blood at the time of immunization (Fig. 3A, left). Pups that were suckled on a MSP1\textsubscript{19}-immunized mother and were immunized at 2 wk of age did not produce MSP1\textsubscript{19} IgG2a\textsuperscript{b}-specific Abs in response to immunization with MSP1\textsubscript{19}, at the same time as the age-matched pups suckled on a nonimmune mother (Fig. 3A). IgM production was also inhibited in the pups suckled on an MSP1\textsubscript{19}-immune mother in response to the first immunization, whereas in the age-matched pups suckled on a nonimmune mother IgM was produced (data not shown). The pups born to the immune mother started to produce detectable MSP1\textsubscript{19}-specific Abs on day 38. These Abs were probably generated in response to the boost that they received 21 days after the initial immunization. The total amount of MSP1\textsubscript{19}-specific IgG2a\textsuperscript{b} produced in response to MSP1\textsubscript{19} immunization in the first 50 days was significantly different between 2-wk-old pups suckled on an MSP1\textsubscript{19}-immune mother and pups suckled on a nonimmune mother (p = 0.002).

Pups that received their first immunization at 3 wk of age and that were suckled on an immune mother also experienced a delay in MSP1\textsubscript{19}-specific IgG2a\textsuperscript{b} Ab production; furthermore, the amount of MSP1\textsubscript{19}-specific IgG2a\textsuperscript{b} Ab produced by the pups suckled on an immune mother was significantly less by day 50 than the pups suckled on a nonimmune mother (p = 0.006) (Fig. 3B). However, there was no significant difference in the amount of MSP1\textsubscript{19}-specific IgG2a\textsuperscript{b} produced in the first 50 days when comparing pups first immunized at 4, 6, or 8 wk of age and suckled on MSP1\textsubscript{19} immune and nonimmune mothers (p = 0.199, 0.166, and 0.094, respectively; Fig. 3, C–E).

The pups suckled on a nonimmune mother and immunized at 2 and 3 wk of age took longer to produce a detectable MSP1\textsubscript{19}-specific IgG2a\textsuperscript{b} response (Fig. 3, A and B) compared with the pups that received their immunization at 8 wk of age (Fig. 3E). Because there were no Abs present in these pups at the time of immunization, the delay may be attributed to the less developed immune system in the 2- and 3-wk-old pups compared with the 8-wk-old pups.

When examining all mice in all groups, there was a significant inverse correlation between the total amount of MSP1\textsubscript{19}-specific IgG present at the time of immunization and the total amount of MSP1\textsubscript{19}-specific IgG2a\textsuperscript{b} (units as defined in Materials and Methods) produced in response to immunization within the first 50 days (r = -0.710, p < 0.001). These data suggested that the presence of high titer maternally derived MSP1\textsubscript{19}-specific Ab present in the pups at the time of immunization interfered with the pups’ ability to produce MSP1\textsubscript{19}-specific Abs.

Effect of maternally derived P. yoelii-specific Abs on the immune response to MSP1\textsubscript{19} immunization

Because we observed that passively transferred P. yoelii HIS had an effect on the Ab response to MSP1\textsubscript{19} immunization in adult mice, we decided to examine the effect of P. yoelii-specific Abs on the response to MSP1\textsubscript{19} immunization in infant mice of various ages. Both female BALB/c mice that had completed the P. yoelii infection and cure regimen and naive female BALB/c mice were bred with naive C57BL/6 males to produce F1 cross-bred pups. These litters of pups were given an initial immunization with MSP1\textsubscript{19} in CFA at 2, 3, 4, or 6 wk of age. This was followed by a boost of MSP1\textsubscript{19} in IFA 3 wk later. At each time point, a litter suckling on an immune mother and a litter suckling on a naive mother were immunized. Total MSP1\textsubscript{19} IgG and IgG2a\textsuperscript{b} Ab in the pups’ sera was measured by ELISA; the production of IgG2a\textsuperscript{b} Ab was indicative of the pups’ Ab response after immunization.

All pups suckled on P. yoelii-immune mothers, irrespective of their age at the time of first immunization, had MSP1\textsubscript{19}-specific...
IgG in their circulation on the day that they were immunized (Fig. 4, left). Despite the presence of these Abs, there was no delay in the MSP119-specific IgG2a b Ab response of pups suckling on P. yoelii-immune mothers when compared with pups of the same age suckling on nonimmune mothers (Fig. 4, right). There was also no significant difference in the amount of MSP119-specific IgG2a b Ab produced in the first 50 days by pups suckled on a MSP119-immune mothers with that by pups suckled on a nonimmune mother for each age group.

Fine specificity of the maternally derived Abs

It was necessary to establish whether maternally derived Abs were interfering with the pups’ Ab response by blocking access to a critical B cell epitope within MSP119. This was determined by performing competition ELISAs examining the ability of the pups’ sera taken on the day of immunization (containing only maternally derived Abs) to inhibit the binding of mAb302 to MSP119. Abs in the sera of pups suckled on MSP119-immune mothers were the most effective at inhibiting the binding of mAb302 to MSP119 compared with their age-matched counterparts suckled on nonimmune mothers (Fig. 5A). This inhibition was the highest in the pups immunized at 2 and 3 wk of age and decreased both as the age of the pup increased and as the level of maternally derived MSP119-specific Abs decreased. In contrast, Abs in the sera of pups suckled on P. yoelii-immune mothers were not very effective at inhibiting the binding of mAb302 to MSP119 compared with the pups suckled on nonimmune mothers (Fig. 5B). These pups had only very low levels of MSP119-specific Abs at the time of immunization (Fig. 4).

The 2-wk-old pups that were unable to produce MSP119-specific Abs in response to immunization had high levels of Abs at the time of immunization that were the most effective at inhibiting the binding of mAb302 to MSP119. Abs in the sera of pups suckled on MSP119-immune mothers were the most effective at inhibiting the binding of mAb302 to MSP119 compared with their age-matched counterparts suckled on nonimmune mothers (Fig. 5A). This inhibition was the highest in the pups immunized at 2 and 3 wk of age and decreased both as the age of the pup increased and as the level of maternally derived MSP119-specific Abs decreased. In contrast, Abs in the sera of pups suckled on P. yoelii-immune mothers were not very effective at inhibiting the binding of mAb302 to MSP119 compared with the pups suckled on nonimmune mothers (Fig. 5B). These pups had only very low levels of MSP119-specific Abs at the time of immunization (Fig. 4).

The 2-wk-old pups that were unable to produce MSP119-specific Abs in response to immunization had high levels of Abs at the time of immunization that were the most effective at inhibiting the binding of mAb302 to MSP119. As both the level of MSP119-specific Abs and the level of inhibition decreased, the ability of pups to produce Abs in response to MSP119 improved. These data suggest...
that the epitope recognized by mAb302 is the B cell epitope critical for production of MSP1_{19}-specific Abs following MSP1_{19} immunization.

**Effect of MSP1_{19}-specific Abs on the proliferative response to MSP1_{19} of T cells from infant mice**

The pups’ lower Ab response, in the presence of maternally derived Abs, could reflect an effect on T or B cells (or both) of the pup. To determine whether maternally derived MSP1_{19}-specific Abs had an effect on the proliferative T cell responses to MSP1_{19} in the pups, 2- and 8-wk-old BALB/c × C57BL/6 F_{1} pups born to MSP1_{19}-immune or naive mothers were immunized with 50 μg of MSP1_{19} in CFA in their hind footpads. The 2-wk-old pups consisted of four mice in each group, and the 8-wk-old pups consisted of three mice in each group. The MSP1_{19}-specific Ab titers in these groups of mice at the time of immunization were similar to the Ab titers of the mice immunized previously. Lymph node cells from individual mice were stimulated with either MSP1_{19}, PPD, or Con A.

Little difference in the T cell-proliferative responses to MSP1_{19} was detected in the 2-wk-old pups born to and suckled on MSP1_{19}-immune or born to and suckled on naive mothers (Fig. 6A). Similarly, there was little difference in the T cell-proliferative responses to MSP1_{19} when comparing the 8-wk-old pups suckled on MSP1_{19}-immune or naive mothers (Fig. 6B). The slightly higher T cell proliferation to MSP1_{19} in the 8-wk-old pups compared with the 2-wk-old pups may reflect a difference in the maturity of the immune system at the ages when the pups were immunized. The similarity in the proliferative T cell responses between the 2-wk-old pups suckled on MSP1_{19}-immune mothers and naive mothers suggested that the presence of maternally derived MSP1_{19}-specific Abs had no effect on the proliferative responses of T cells from the infant pup. These results indicated that the inability of 2-wk-old pups suckled on a MSP1_{19}-immune mother to produce Abs in response to immunization with MSP1_{19} could not be attributed to the failure of Ag presentation to T cells.

**Determining the phenotype and origin of the cells proliferating in response to MSP1_{19} in infant mice**

Studies have shown that maternal cells are transferred from the mother to her pups (35, 36). We decided to determine the phenotype and origin of proliferating T cells in the pups and to exclude that the T cell responses seen in the pups were not due exclusively...
to proliferation of maternal cells transferred to the pup. Prolifera-
tion assays used a fluorescent dye (PKH26; Refs. 37 and 38) to
identify proliferating cells. The use of this dye allowed for further
labeling of the cells with cell surface markers to assess the phe-
notype of the proliferating cells using flow cytometry.

Cells were removed from the popliteal and inguinal lymph
nodes of C57BL/6 (H-2 b ) adult mice, BALB/c (H-2 d ) adult mice
and 2-wk-old F 1 cross-bred pups (H-2 b /H-2 d ) that were born to
MSP1 19 -immune or nonimmune mothers and that had been im-
uminated with 50 μg of MSP1 19 8 days earlier. Cell suspensions
were prepared and stained with PKH26 and then incubated for 96 h
with MSP119 , Con A, or PPD. After incubation, the cells were
stained with Abs speci
cific for CD3 and H-2 b and prepared for
flow cytometry. Proliferating cells staining positive for H-2 b would be
cells of pup origin.

Cells from all mouse groups within the populations of prolifer-
ating cells (see Materials and Methods) expressed similar levels of
CD3 (data not shown). Con A and PPD-induced proliferating cells
from pups born to both immune and nonimmune mothers ex-
pressed levels of H-2 b approximately midway between levels ex-
pressed by the parental haplotypes (BALB/c, H-2 d ; and C57BL/6,
H-2 b ) (Fig. 7, A and B). Although levels of H-2 b expressed on
yMSP1 19 -induced proliferating cells from F1 pups were lower than
levels of H-2 b expressed on Con A and PPD-induced cells, these
lower levels were significantly different from levels expressed on
BALB/c cells (p = 0.04 and p < 0.001 for pups suckled on im-
mune and nonimmune mothers, respectively) (Fig. 7), indicating
that cells of pup origin were proliferating. Interestingly, when ex-
amining the level of H-2 b staining in the pups as a percentage of
the difference between the levels expressed by the parental mouse
haplotypes, there was a significant difference when comparing the
pups suckled on a MSP1 19 -immune mother and the pups suckled
on a nonimmune mother (p = 0.029). Although these data indicate
that T cells of pup origin are proliferating in response to MSP1 19 ,
the data also suggest that both 1) maternal T cells are contributing
to the proliferation of T cells from 2-wk-old pups and 2) the
MSP119 -immune status of the mother has had an effect on the
population of proliferating cells.

Discussion
The development of an effective vaccine against malaria, particu-
larly for young children and pregnant women, is crucial. It is well
known that maternally derived Abs can be transferred across the
placenta to the fetus (39), and these Abs are present in the infant
for the first few months after birth. Additionally, it is known that
these specific Abs may interfere with the infant’s ability to produce
Abs in response to vaccination with the same Ag (2–4). Because
these Abs persist for only a short time after birth (40), vaccination
of infants against infectious diseases such as malaria may be re-
quired at a time when the maternally derived Abs are still present
and may interfere with the development of a response to vaccina-
tion in the infant.

After malaria infection, it has been shown that the levels of
maternally derived anti-MSP1 19 IgG in the infant were correlated

FIGURE 5. Ability of pup sera containing maternally derived Abs to inhibit the binding of
mAb302 to MSP1 19 . Sera from pups suckled on
MSP1 19 -immune mothers and age-matched pups
suckled on nonimmune mothers (A) and P. yoelii-immune mothers and age-matched pups suckled
on nonimmune mothers (B) were taken on the day of immunization and assessed for their
ability to interfere with the binding of mAb302 to MSP1 19 . Different groups of pups were used
as age-matched litters for the MSP1 19 experiment and the P. yoelii experiment. +, Mean percent inhibition of serum from five to eight pups per group.
with a decrease in placental malaria and a delay in the infant’s first detected infection (23). Still to be determined is the effect that maternally derived Abs generated as a result of vaccination have on the infant’s ability to respond to vaccination. Using a rodent model of malaria, the effect of maternally derived whole parasite-specific and MSP119-specific Abs on the pups’ ability to respond to immunization with MSP119 was examined.

The presence of *P. yoelii* and MSP119-specific Abs in adult mice at the time of MSP119 immunization interfered with the Ab response following immunization (Fig. 1). The greatest interference was in the mice that received sera containing MSP119-specific Abs, indicating that the specificity of the Abs played a major role. Additional experiments determined that the presence of MSP119-specific Abs (and to a lesser extent *P. yoelii*-specific Abs) at the time of MSP119 immunization affected the number of B cells that was activated and subsequently secreted MSP119-specific Abs (Fig. 2).

With the use of a mother-pup model, the presence of maternally derived Abs was examined to determine whether they could interfere with the pups’ ability to respond to MSP119 immunization. It was found that MSP119-specific Abs interfered with the development of an Ab response following immunization (Fig. 3) and that this appeared to be related to the level of Abs present in the pup at the time of immunization. It affected not only the time until Ab production but also the total amount of Ab produced in response to the immunization.

In the pups, there was no interference in the development of an anti-MSP119 Ab response due to maternally derived *P. yoelii* (whole parasite)-specific Abs (Fig. 4). This was different from the results observed in the adult mice. The interference observed in the adult mice was most likely the result of greater levels of MSP119-specific Ab titers in the *P. yoelii*-specific HIS than in the pups that received *P. yoelii*-specific Abs from their mother. This suggested that the degree of interference with immunization was due not only to specificity but also to the level of specific Ab.

Because the ability of maternally derived Abs to interfere with MSP119 immunization was inversely correlated with the amount of Ab present at the time of immunization, it was most likely that the Abs were blocking access to a single critical B cell epitope. By examining the ability of these Abs to inhibit the binding of mAb302 to MSP119 (Fig. 5), it was found that the maternally derived Abs recognized the same epitope as mAb302 and that the
level of inhibition was influenced by the Ab titer. This suggested that the maternally derived Abs were most likely blocking access to the immunodominant B cell epitope, thereby preventing the pups from producing their own Ab response. A decrease in the level of maternally derived Abs in the pups would give the pups’ B cells access to the epitope, resulting in the production of MSP119-specific Abs. In a recent study, we observed that after malaria infection, maternally derived MSP119 and *P. yoelii*-specific Abs directed the pups’ Ab response toward an alternate epitope (30). In the presence of maternally derived Abs, the development of an Ab response in the pups after parasite challenge compared with the complete inhibition of an Ab response after MSP119 immunization may be explained by the availability of alternate epitopes. In the context of parasite infection, when the epitope recognized by maternally derived Abs and mAb302 is blocked, alternate B cell epitopes are available for the pups to respond to. After MSP119 immunization, only the B cell epitope recognized by the maternally derived Abs and mAb302 is available to the pup, with recognition by the pup correlated with the level of maternally derived Ab present. The development of an infant vaccine containing an alternate B cell epitope to that of the adult vaccine is the most obvious approach to overcoming interference by specific maternally derived Abs.

Studies on the effect of specific Ab on T cell proliferation in the pup showed that MSP119-specific T cell proliferation was the same in the presence or absence of MSP119-specific Abs (Fig. 6). This indicated that the Ag processing and presentation to T cells was unaffected by the presence of specific maternally derived Ab. Additionally, it was shown that although T cells of pup origin could proliferate in response to MSP119, the level of H-2b staining was less in the pups suckled on immune mothers (Fig. 7). We therefore cannot exclude the possibility that the population of proliferating cells contained a proportion of maternal cells and that maternal immunity negatively affected the response of T cells of pup origin to MSP119.

The data presented in this paper offers a possible alternative to Siegrist’s hypothesis on the influence of maternal Abs on neonatal vaccine responses (6). It is thought that maternally derived Abs bind to the immunodominant epitope on the Ag, thereby preventing the infant’s B cells from binding to the same epitope. This would explain the effect of specific Ab on the number of B cells produced after immunization given that fewer B cells would have access to the epitopes in the presence of maternally derived/passively administered Ab. Following binding of maternally derived Ab to Ag, the immune complexes are taken up by non-B APCs,
processed, and then presented to the T cells. The T cell-proliferative data presented in this paper confirmed that Ag presentation was still occurring in the presence of maternally derived Abs despite the inhibition of Ab responses. However, the data suggest that maternal T cells were contributing to this proliferation; furthermore, we cannot discount that the immune status of the mother does have an effect on proliferation of specific T cells of pup origin.

The WHO Collaborating Centre for Neonatal Vaccinology has done considerable work with hepatitis virus (41), measles virus (8), and respiratory syncytial virus (42), examining the effect of maternally derived Abs on vaccination. Once it had been established that maternal Ab-mediated inhibition of infant Ab responses observed in humans was reproducible using a murine model, various vaccine systems were tested. The Ab response to live attenuated vaccines in pups was very susceptible to the influence of maternal Abs, presumably because they can affect the in vivo replication of the virus (8). It was also observed, however, that the Ab response in pups to nonlive vaccines was equally affected by the presence of high titer maternal Abs. Interestingly, although they found that maternally derived Abs inhibited the production of Abs to the vaccine in the offspring, there was not a complete inhibition of the immune response, with vaccine-induced T cell proliferation, cytokine production, and CTL responses unaffected. They also observed that this inhibition of Ab responses in the presence of maternally derived Abs could be overcome if one dose of the live attenuated measles vaccine was followed by a second dose of the vaccine as early as 10 days after priming. In the present study, the 2-wk-old pups that failed to make MSP119-specific IgG2a, also failed to produce MSP119-specific IgM in response to the first immunization (compared with the age-matched controls that produced both IgM and IgG2a); therefore, it seems unlikely that priming occurred in these mice. We cannot completely exclude this possibility, and further studies may address this phenomenon in our system.

DNA vaccines have been successful at circumventing the influence of maternally derived Abs on the induction of an immune response in pups born to immune mothers (43, 44). However, DNA vaccination is not always successful at immunizing neonates in the presence of maternal Abs, presumably because they can affect the in vivo replication of the virus (8). It was also observed, however, that the Ab response in pups to nonlive vaccines was equally affected by the presence of high titer maternal Abs (45). It has been postulated that the capacity of DNA vaccines to circumvent maternal Ab-mediated inhibition depends on whether in vivo Ag production outlasts the persistence of inhibitory titers of maternal Abs in the offspring (8). It has been observed that the i.m. delivery permits long-lived low level expression of foreign protein (46, 47), and this expression may persist longer than the maternally derived Abs, thereby permitting induction of an Ab response.

As outlined above, the observation that maternally derived specific Abs interfere with the development of an Ab response in the offspring has been observed in a number of different systems. Murine mother-pup studies directly pertinent to the development of a malaria vaccine however, have focused only on using the whole parasite or a subunit vaccine candidate with a mode of protection that is dependent on the induction of CD8 T cell responses. This present study focuses on MSP119, a leading malaria subunit vaccine candidate, the protective immunity of which is dependent on the presence of high titer Abs, and the data have very important implications for immunization strategies. Immunizing women of child-bearing age with a malaria subunit vaccine candidate may facilitate the transfer of protective Abs to her fetus, which will act to protect the infant in the first few months of life. These data indicate, however, that if high levels of maternally derived Ab specific for a malaria subunit vaccine candidate are present in the infant, successful immunization of the infant with the same vac-

cine may not be efficient. Different approaches to infant vaccination need to be investigated to ensure effective vaccination against malaria in early life.

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