CHARACTERIZATION OF A HUMAN REFERENCE STANDARD FOR ANTIBODY TO PLASMODIUM FALCIPARUM MEROZOITE SURFACE PROTEIN 142

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Abstract. Volunteers vaccinated with a candidate malaria vaccine containing merozoite surface protein 142 (MSP-142) exhibit antibodies to MSP-142 that are measured by enzyme-linked immunosorbent assay (ELISA). The purpose of this study was to make a human reference standard for MSP-142 antibody measured in absolute quantity units. Immobilized metal affinity chromatography was used to determine the amount of MSP-142 antibody in this plasma pool. Hexahistidine-tagged MSP-142, antigen adsorbed to nickel-chelating resin was used to capture MSP-142 antibody from the plasma pool. The intact MSP-142 antibody-antigen complexes were eluted and total IgG was measured by an ELISA standardized against purified human IgG. In this way, the human reference standard was determined to contain 48.3 μg/mL of MSP-142 antibody. This reference standard may be useful as a quantitative working standard for measuring MSP-142 antibody response in future vaccine clinical trials involving MSP-1.

INTRODUCTION

The major merozoite surface protein 1 (MSP-1) is one of the most widely studied parasite antigens from the erythrocyte stage of infection by Plasmodium falciparum. It is secreted as a 195-kD protein that is proteolytically cleaved to form polypeptide fragments of various molecular masses. The 42-kD C-terminal fragment (MSP-142) is further processed to a 19-kD C-terminal fragment (MSP-142) that remains on the merozoite surface at the time of erythrocyte invasion. MSP-142 consists of two epidermal growth factor-like domains with a total of six disulfide bridges. The tertiary structure that is formed contains several distinct epitopes that most likely comprise the immunodominant region of the MSP-1 molecule.

Both MSP-142 and MSP-142 are leading vaccine candidates for the blood stage of P. falciparum. Multiple animal studies including those on mice and primates support the protective efficacy of vaccine candidates based on the 19-kD or 42-kD C-terminus fragment of MSP-1. Some studies suggest a correlation between the degree of antibody response and the level of protection. In human studies using blood samples collected from malaria-endemic areas, high antibody titers to MSP-1 are associated with resistance to clinical malaria and lower parasitemia. Antibodies to MSP-142 from malaria-resistant humans inhibit in vitro parasite invasion of red blood cells. Two human safety trials in malaria-naïve subjects using early versions of MSP-1 candidate vaccines have shown either poor immunogenicity or unacceptable safety issues. Other promising next-generation MSP-142 vaccine candidates have been purified for use in human clinical trials.

In these and other prior studies, antibody levels to MSP-1 have typically been measured in relative quantity units usually using different enzyme-linked immunosorbent assay (ELISA) systems. A fully quantitative measure of antibody response would allow better comparison of immunogenic data from different studies at various centers. This need may become more pressing in the future, given the likely increase in the number of human vaccine trials using various formulations of MSP-1. The purpose of this study was to develop a human reference standard for measuring antibody levels to MSP-142 in absolute quantity units. This reference standard could serve as a quantitative working standard for future ELISAs assessing MSP-142 antibody response to vaccine.

MATERIALS AND METHODS

Human reference standard. Human plasma samples were available from a completed clinical trial at the Walter Reed Army Institute of Research that used a candidate malaria vaccine containing MSP-142 as one of its components (Cummings J and others, unpublished data). Twenty-two separate samples were obtained for which the corresponding serum had previously undergone ELISA measurement and been found to have high titers of antibody to MSP-142 in response to vaccine. Titers, defined as the dilution required to attain an optical density (OD) of 1.0, ranged from 52,227 to 509,680 with a mean of 135,267. Equal volumes of each sample were combined to form a pool of MSP-142 antibody-positive human plasma that served as the human reference standard for further characterization. A separate pool of MSP-142 antibody-negative plasma was made by combining 21 pre-vaccination samples having low MSP-142 titers (none greater than 37.5).

Immobilized metal affinity chromatography (IMAC). Nickel-nitrioltriacetic acid (NTA) Superflow (Qiagen, Valencia, CA) was equilibrated with equilibration buffer (E buffer; 0.01 M sodium phosphate, pH 7.4, 1 M sodium chloride, 2% Tween 80 [v/v], and 0.01 M imidazole). Three 0.5-mL microfuge tubes were set up, each containing 100 μL of E buffer. Hexahistidine-tagged (6XHis-tagged) MSP-142 was obtained from a stock previously purified containing 0.95 mg/mL of antigen. Thirty microliters of 6XHis-tagged MSP-142 was added to tubes 1 and 3 while 30 μL of E buffer was added to tube 2. All tubes were rocked at room temperature for 20 minutes in a batch-binding method and centrifuged at 15,000 rpm for 5 minutes. One hundred microliters of supernatant was collected from each tube. The resin was washed with 300 μL of E buffer four times in sequence. Each wash step was done in a fashion similar to the batch-binding method except that tubes were rocked only briefly to mix resin and buffer, and 300 μL of wash solution was collected after each wash step. The antibody-positive human reference standard was diluted 1:100 in E buffer and 100 μL of this was added to tubes 1 and 2 (for the equivalent of
The antibody-negative pool was also diluted 1:100 and 100 μL was added to tube 3. All tubes were rocked at room temperature for 20 minutes and centrifuged at 15,000 rpm for 5 minutes. One hundred microliters of supernatant was collected from each tube. The resin was washed with 300 μL of E buffer six times in sequence. Elution of the antibody-antigen complex from the nickel resin was performed by adding 300 μL of 0.01 M sodium phosphate, pH 8.0, 1 M sodium chloride, 2% Tween 80 [v/v], and 0.5 M imidazole. The tubes were rocked at room temperature for 20 minutes and centrifuged at 15,000 rpm for 5 minutes. Three hundred microliters of eluate was collected from each tube (yielding eluates 1, 2, and 3). To allow testing of the remaining nickel resin for retained antibody-antigen complexes, the resin was washed with 300 μL of E buffer four times in sequence, leaving 100 μL of nickel resin after the final wash.

**Enzyme-linked immunosorbent assay.** The concentration of IgG in eluates was measured by immunocapture ELISA based on a previously published method with modifications. Ninety-six-well, flat-bottom microtiter plates (Nunc Maxisorp, no. 439454; Nalge Nunc International, Rochester, NY) were used. Each well was coated with 100 μL of affinity-purified polyclonal goat anti-human IgG (no. 13382; Sigma, St. Louis, MO) diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6 (no. C3041; Sigma) at a concentration of 1.25 μg/mL. The plates were incubated overnight at 4°C. Each well was blocked for one hour at room temperature with 220 μL of a solution of phosphate-buffered saline (PBS), pH 7.4, 5% skim milk (no. 211915; Becton Dickinson, Franklin Lakes, NJ) and 0.5% Tween 20. The plates were washed four times with a solution of PBS and 0.05% Tween 20 on an automatic plate washer (Skatron Washer 300; Skatron Instruments, Tranby, Norway). For each eluate, 100 μL of eight two-fold serial dilutions in duplicate starting at 1:5 were incubated in appropriate wells for one hour at room temperature. The diluent used was PBS, pH 7.4, 5% skim milk, and 0.5% Tween 20. Every plate contained 100-μL samples of eight two-fold serial dilutions in duplicate of a quantitative standard of purified human IgG (no. I2511; Sigma) over the concentration range of 200 ng/mL to 1.56 ng/mL. After incubation, the plates were washed four times as above. One hundred microliters of horseradish peroxidase–conjugated polyclonal goat antihuman IgG (no. 2040-05; Southern Biotechnology, Birmingham, AL) diluted to 1:3,000 was added to each well. The plates were incubated for one hour at room temperature and washed four times as above. One hundred microliters of developing solution consisting of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide:1:1 (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well and incubated for one hour at room temperature. The reaction in each well was stopped with 10 μL of 20% sodium dodecyl sulfate (SDS) solution. Absorbance was measured at 414 nm using a Vmax microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

Supernatant and subsequent wash solution from the antibody-binding step in IMAC were tested for unbound antibody to MSP-1<sub>42</sub> by an ELISA similar to the one described above except for the following changes. The coating antigen used for each well was 100 μL of MSP-1<sub>42</sub> at a concentration of 0.35 μg/mL. In addition, the sample incubation was done with eight two-fold serial dilutions starting at 1:10. Every plate contained 100-μL samples of eight two-fold serial dilutions of the antibody-positive human reference standard starting at 1:800. All other aspects of the ELISA were identical to those above.

**Sodium dodecyl sulfate polyacrylamide–gel electrophoresis (SDS-PAGE).** SDS-PAGE (Invitrogen, Carlsbad, CA) followed by Coomassie Blue R-250 total protein staining (Bio-Rad Laboratories, Hercules, CA) was used to assess the supernatant and subsequent wash solution from the binding of 6XHis-tagged MSP-1<sub>42</sub> to nickel resin for any unbound MSP-1<sub>42</sub>. SDS-PAGE was also used to test the residual nickel resin at the end of IMAC for any retained MSP-1<sub>42</sub>. It was performed under non-reducing conditions with Tris-glycine buffer (Invitrogen).

**Data analysis.** The ELISA data was analyzed using Softmax® Pro Version 4.6 software (Molecular Devices Corporation). For each sample, linear regression was performed on three consecutive dilutions with an OD ≤ 1.5 beginning with the OD closest to 1.5. The dilution factor to attain an OD of 1.0 was then calculated from the linear regression equation. Since each sample was run in duplicate, the mean of the dilution factor at OD 1.0 for each duplicate was used for all calculations. Duplicates with a coefficient of variation (CV) greater than 10% were excluded. Every plate included serial dilutions of purified human IgG of known concentration on which linear regression was performed. This purified IgG was used to create standard curves for every plate to normalize other samples on that plate. The IgG concentration that was obtained for each eluate was multiplied by 400 to arrive at the IgG concentration for undiluted plasma (since approximately 400 μL of eluate volume came from an original plasma volume of 1 μL in IMAC).

Three repeat IMAC experiments were performed. For each experiment, the quantity of antibody to MSP-1<sub>42</sub> was calculated by subtracting IgG in eluate 2 (background binding) from IgG in eluate 1 (specific MSP-1<sub>42</sub> antibody-antigen binding plus background binding). The IgG in eluate 3 served as a control demonstrating binding of antibody-negative plasma to verify the accuracy of background binding in eluate 2. The results from the three IMAC experiments were averaged to obtain a final value for antibody to MSP-1<sub>42</sub> in the human reference standard.

**RESULTS**

Using the mean from three repeat experiments, the concentration of antibody to MSP-1<sub>42</sub> in the human reference standard was determined to be 48.3 μg/mL with an SD of 15.6 μg/mL (CV = 32.3%). A summary of the results is shown in Table 1.

Figure 1 shows data from a typical anti-human IgG-coated ELISA plate illustrating the linearity of serial dilutions near an OD of 1.0. For all linear regressions performed according to the procedure described in the Materials and Methods, all regression coefficients were ≥ 0.98. Figure 2 shows data from a typical anti-human IgG-coated ELISA plate illustrating graphical parallelism in the linear range for all eluates and the purified IgG standard when normalized to OD equivalent dilutions (arbitrary units).

The ELISAs using MSP-1<sub>42</sub> antigen-coated plates done on the supernatant from the antibody-binding step and subsequent wash step in IMAC showed no significant quantities of
unbound antibody to MSP-142 when compared with the antibody-positive human reference standard run on the same plate (Figure 3). This indicated that nearly all of the antibody to MSP-142 in the reference standard was captured by the MSP-142 antigen bound to the nickel resin. Of note, this MSP-142 antigen-coated ELISA system would only be valid if a significant amount of competing MSP-142 antigen were not present in the supernatant being tested. A competition ELISA using as the competitor the supernatant from the antibody-binding step from tube 3 in IMAC confirmed the lack of significant competing antigen in the supernatant.

The SDS-PAGE analysis performed on the nickel resin remaining at the end of IMAC demonstrated the absence of any MSP-142. This indicated that essentially all MSP-142 antigen (and by inference all MSP-142 antibody-antigen complexes) had been eluted from the nickel resin during the elution step. In addition, SDS-PAGE performed on the supernatant from the binding of MSP-142 antigen to nickel and subsequent wash step demonstrated the absence of any MSP-142. This indicated that nearly all MSP-142 antigen was bound to the nickel resin and remained bound until the elution step.

**DISCUSSION**

In this study, we quantitated the amount of MSP-142 antibody in a human reference standard consisting of pooled high-titer plasma obtained from a clinical trial of an MSP-142 vaccine candidate. The calculated value of 48.3 μg/mL provides a measure of the amount of specific antibody produced during a high-titer response to MSP-1 vaccine administration. By using this human reference standard as a quantitative working standard in ELISAs for future unknown samples, we should be able to assign absolute quantities to antibody responses instead of reporting titer values alone.

The quantitation procedure described here avoided a potential problem inherent in many antibody affinity purification methods, namely that of adversely impacting antibody function during the purification process. We avoided the problem by taking advantage of the relatively mild elution conditions present in IMAC and using a strategy of backward extrapolation of antibody quantity rather than direct purification of antibody. In so doing, we avoided the harsh conditions that might be needed to separate antibody from
antigen, instead eluting the intact antibody-antigen complex from the nickel resin and measuring antibody within the intact complex. This measurement was then used to infer the antibody quantity in the original reference standard. Our procedure was therefore a method of quantitation rather than a method of purification.

A necessary assumption in this quantitation strategy was that the final eluate contained all of the antibody to MSP-142 present in the original plasma. There needed to be complete binding of antibody to the IMAC system and complete release of antibody-antigen complexes during elution. To meet these conditions, the amount of antigen added to the nickel resin was limited to an amount significantly below the binding capacity (only 3–6% of binding capacity). In addition, the amount of plasma added to the nickel resin was titrated such that no significant amount of antibody would be present in the supernatant fluid from the antibody-binding step. These conditions were confirmed to be present by an MSP-142 antigen-coated ELISA performed on the relevant supernatant and by an SDS-PAGE performed on the residual nickel resin (see Results).

A potential obstacle in measuring antibody within intact antibody-antigen complexes was steric hindrance during the anti-human IgG-coated ELISA due to the size and conformation of the complex. This situation would be worsened if cross-linkages were to form among the complexes. The likelihood of such cross-linkage formation, however, was minimized by the relative overabundance of antigen as compared with antibody in the ELISA. Furthermore, individual antibody-antigen complexes did not seem to cause significant steric hindrance, as indicated by the existence of a linear section in all the ELISA curves for all eluates. In addition, the graphical parallelism illustrated in Figure 2 further supported the equivalence of the ELISA systems measuring free antibodies versus antibody-antigen complexes. A final bit of confirmation was provided by the fact that halving the concentration of anti-human IgG coating in the ELISA did not increase the calculation of IgG concentration.

The use of IMAC did result in a significant level of background binding of IgG to nickel. Various manipulations in binding buffer stringency were made to decrease this background binding without negatively impacting MSP-142 antigen binding to nickel or MSP-142 antibody binding to antigen. The conditions described in the Materials and Methods represent a compromise of these manipulations. Much of the observed background binding was likely due to the natural affinity of immunoglobulin for nickel. A probable result of this background binding was the relatively large standard deviation of the final quantitation value for antibody to MSP-142. Our quantitation method, therefore, did not yield excellent precision, which was most likely due to the IMAC steps. Nevertheless, this method should be able to distinguish among significant differences in antibody quantity (i.e., differences of greater than a factor of 2), which should be sufficient for many applications of this data.

An assumption made in using a reference standard with an absolute quantity value is that the average avidity of the polyclonal antibody response in an unknown sample is similar to that in the reference standard. This may be a reasonable assumption if the unknown sample is from a clinical trial using a vaccine candidate similar to the trial from which the pooled reference standard was obtained. If the average antibody avidity in an unknown sample were significantly different, then using this reference standard in an ELISA would give an “effective antibody concentration” combining the effects of quantity and avidity rather than quantity alone. This latter situation would not be dissimilar from reporting data in ELISA titer units.

Absolute quantitation of antibody level may also yield information about the relative avidities of different populations of polyclonal antibodies to MSP-142. If two different sample pools from two different clinical trials had similar titers by the same ELISA, but were significantly different in absolute quantity of antibody, an inference could be made that the average antibody avidity of the lower quantity pool was greater than that of the higher quantity pool. This method could be used, for example, to compare the avidities of two positive controls from two different clinical trials.

Total MSP-1 antibody quantity is unlikely to be the only determinant of resistance to merozoite invasion. Avidity and fine specificity of antibodies in addition to other undetermined factors are likely to play an important role in immunity. Antibodies with overlapping specificities to MSP-1 epitopes may either play a protective role against parasite invasion or conversely promote invasion by blocking the protective ability of the former type of antibody. It has been suggested that evaluation of MSP-1-based vaccine trials should include assays that look at fine specificity of antibodies to MSP-1 in addition to total titers. Given that truly reliable immune correlates of protection have yet to be identified, absolute quantitation of total antibody to MSP-1 provides useful information that improves the ability to compare immunogenic responses from different MSP-1-based studies.

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