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Synthetic Low-Toxicity Muramyl Dipeptide and Monophosphoryl Lipid A Replace Freund Complete Adjuvant in Inducing Growth-Inhibitory Antibodies to the *Plasmodium falciparum* Major Merozoite Surface Protein, gp195

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The *Plasmodium falciparum* major merozoite surface protein (gp195) is a protective antigen against lethal malaria. However, increasing evidence indicates that the efficacy of a malaria vaccine will require a strong adjuvant that is safe for human use. We compared the efficacies of two low-toxicity synthetic immunomodulators, B30-MDP (a lipophilic muramyl dipeptide derivative) and LA-15-PH (a synthetic equivalent of monophosphoryl lipid A), with that of Freund complete adjuvant (FCA) in eliciting an antibody response to gp195. Rabbits were immunized with native gp195 and B30-MDP, LA-15-PH, or the two in combination, with liposomes as the vehicle. Aluminum hydroxide and FCA were used as reference adjuvants. Results showed that adjuvant formulations based on B30-MDP alone or in combination with LA-15-PH induced high antibody titers to gp195, as compared with FCA. LA-15-PH alone was less effective. Aluminum hydroxide induced significantly lower antibody titers. The functional activity of the rabbit anti-gp195 antibodies induced by different adjuvants was evaluated in an in vitro parasite growth inhibition assay previously shown to correlate with anti-gp195 immunity in the *Aotus* monkey model. All rabbits immunized with B30-MDP–LA-15-PH and two of three rabbits immunized with B30-MDP alone produced sera that strongly inhibited parasite growth. The degree of growth inhibition was similar to that with FCA. The antibody titers of the rabbits receiving B30-MDP–LA-15-PH strongly correlated with the degree of in vitro growth inhibition. Our findings provided strong evidence that adjuvant formulations based on synthetic B30-MDP and LA-15-PH can replace FCA as adjuvants in stimulating protective immunity specific for gp195.

In the design of synthetic peptide and recombinant polypeptide vaccines, the need for a safe and effective adjuvant has received increasing attention. Although native proteins are good immunogens, subunit vaccines often have reduced immunogenicity. In addition, protective immunity against many pathogens may require the participation of both humoral and cell-mediated immunities, and the induction of these immunities may depend on the administration of a strong adjuvant. At present, aluminum hydroxide (alum) is the only adjuvant approved for human use and is efficacious in a limited number of vaccine systems (3, 25, 31). While studies have shown that alum-type adjuvants can potentiate humoral immunity, they are poor inducers of cell-mediated immunity (6). Recent clinical vaccine trials with malaria sporozoite and blood-stage antigens and with alum as an adjuvant showed that the immunogenicity of these antigens was poor and that the protection afforded by these vaccines was modest (5, 11, 23). These results indicate that stronger adjuvants are needed to improve the overall efficacy of malaria vaccines. Freund complete adjuvant (FCA) is regarded as the most potent stimulator of both parts of the immune response. It remains a challenge to develop an adjuvant formulation with an efficacy similar to that of FCA but lacking its harmful side effects.

Bacterial cell wall derivatives such as muramyl peptides (MDP) and lipid A derivatives (monophosphoryl lipid A, or MPL) are two major classes of immunomodulators currently being investigated as potential human vaccine adjuvants (40). Among the antigens tested with MDP adjuvants are herpes simplex virus type 1 envelope glycoprotein (30), hepatitis B surface antigen (4), human immunodeficiency virus type 1 envelope glycoprotein (29), and simian type D retrovirus (19). MPL has been used in immunogenicity studies for ovalbumin (15), herpes simplex virus proteins (22), Epstein-Barr virus (21), and cholera toxin (1). In malaria vaccine development, MDP derivatives have been tested with blood-stage and circumsporozoite (CSP) proteins (8, 18, 34). MPL purified from *Escherichia coli* has been shown to induce high antibody titers to recombinant CSP antigen (27), and the parent lipid A compound has been shown to enhance antibody responses to synthetic CSP peptides (1).

Previous studies showed that vaccination of *Aotus* and *Saimiri* monkeys with the *Plasmodium falciparum* major merozoite surface protein, gp195, protected these animals against lethal malaria parasite infections (10, 24, 33). However, protection was dependent on the administration of FCA as an adjuvant. Recently, two synthetic, low-toxicity derivatives of MDP and MPL, namely, B30-MDP (lipophilic MDP) and LA-15-PH (synthetic equivalent of MPL), respectively, were shown to induce potent antibody and cell-mediated responses to a variety of antigens, including the influenza A split vaccine and the hepatitis B surface antigen (15, 37–39). The objective of the present study was to evaluate the ability of these two synthetic immunomodulators to potentiate the antibody response to gp195 in rabbits.

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We found that two formulations based on these immunomodulators were as effective as was FCA in eliciting anti-gp195 antibodies which could inhibit parasite growth in vitro, an assay that has been shown to correlate with the induction of protective immunity in gp195-vaccinated monkeys (14). These results provide strong evidence that adjuvant formulations based on B30-MDP and LA-15-PH may be efficacious in a gp195-based vaccine.

MATERIALS AND METHODS

Parasites. The *P. falciparum* Uganda Palo Alto isolate was used throughout this study. Parasites were cultured by established methods (13, 36), and whole parasites were collected by saponin lysis. Synchronized parasite cultures were obtained by a single sorbitol lysis treatment (16).

Parasite gp195. gp195 proteins were isolated from Nonidet P-40 extracts of in vitro-cultured parasites by monoclonal antibody affinity chromatography (32). In brief, saponin-lysed parasites were extracted with 1% Nonidet P-40, and the lysate was clarified by ultracentrifugation. The extracts were passed through a protein G-Sepharose column covalently conjugated with a gp195-specific monoclonal antibody (5.2). After being extensively washed to remove nonspecific binding, the bound proteins were eluted with 0.1 M glycine (pH 2.5) and neutralized with 1 M Tris-HCl (pH 8.0). The purity of the isolated gp195 was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, silver staining, and immunoblotting with gp195-specific monoclonal antibodies. The proteins isolated were the 195-kDa precursor protein and several lower-molecular-mass processing fragments of gp195.

Adjuvant components. The synthetic immunomodulators B30-MDP and LA-15-PH were chemically synthesized and purified as described previously (15, 37, 39). The optimal dose of each immunomodulator for use in rabbits was previously determined to be 200 μg per injection. Liposome components (cholesterol, L-α-phosphatidylcholine dimyristoyl, and dicetyl phosphate) were purchased from Sigma Chemical Co. (St. Louis, Mo.).

gp195 and immunomodulators in liposomes. Multilamellar liposomes were used as vehicles, since it was previously determined that liposomes are better vehicles than are squalene-water emulsions in inducing anti-gp195 antibodies in rabbits (35a). Furthermore, studies have shown that liposomes can reduce the toxicity of lipid A compounds (9, 28). Liposomes were prepared as described by Alving et al. (2), except that the lipids content was increased fivefold, since pilot studies with iodinated gp195 showed that antigen entrapment increased with the packed volume of liposomes. Therefore, 50.5 mg of L-α-phosphatidylcholine dimyristoyl, 21.8 mg of cholesterol, and 4.5 mg of dicetyl phosphate and 200 μg of B30-MDP, LA-15-PH, or B30-MDP and LA-15-PH in combination were dissolved in chloroform-methanol (4:1) and shell evaporated under a vacuum on the wall of a Wheaton 50-ml rotoflask. gp195 (50 μg) in 0.5 ml of borate-buffered saline was added to the rotoflask together with 10 glass beads, and multilamellar liposomes were formed by gentle shaking at 4°C for 30 min. The final emulsion showed an increase in L-α-phosphatidylcholine dimyristoyl from 10 to 50 mM, while the L-α-phosphatidylcholine dimyristoyl/cholesterol/dicetyl phosphate molar ratio remained at 1:0.75:0.11.

Other adjuvants. gp195 in FCA was prepared by emulsifying 50 μg of gp195 with an equal volume of FCA (GIBCO, Grand Island, N.Y.). For subsequent booster immunizations, the amount of mycobacteria in the emulsion was successively halved. gp195 (50 μg) was also adsorbed to alum (Alhydrogel; Accurate Chemical & Scientific Corp., San Diego, Calif.) in accordance with the manufacturer's instructions.

Immunization procedure. A total of 26 New Zealand White rabbits (approximately 5 kg) were used. Rabbits (three to six animals per group) were immunized intramuscularly four times at 4-week intervals, each time with 50 μg of gp195 in different adjuvant formulations as follows: gp195-FCA, gp195–B30-MDP–liposomes, gp195–LA-15-PH–liposomes, gp195–B30-MDP–LA-15-PH–liposomes, gp195-liposomes, gp195-alum, and gp195-saline. Serum samples were collected 1 week before immunization and weekly after each immunization.

Rabbit anti-gp195 antibody assays. Serum anti-gp195 antibodies from rabbits were assayed by an enzyme-linked immunosorbent assay (ELISA) with purified gp195 as the coating antigen at a concentration of 200 ng per well (7). Wells were blocked with 1% bovine serum albumin, and serial dilutions of rabbit sera were incubated in duplicate wells. After seven successive washings with borate buffer, wells were incubated with goat antirabbit immunoglobulin G-peroxidase (Zymed) at a 1/1,200 dilution. Wells were washed and incubated with 100 μl of H_2O_2-ABTS [2,2′–azino–bis(3–ethylbenzothiazoline–6–sulfonate)] (Zymed), and the absorbance was measured with a Dynatech ELISA reader. Endpoint titers were determined from ELISA titration curves, and an optical density of 0.2 was chosen as the endpoint absorbance, a value more than fivefold above the mean absorbance value plus 1 standard deviation of preimmune sera. Anti-gp195 antibodies were also measured by an indirect immunofluorescence assay (IFA) with in vitro-cultured *P. falciparum* schizonts or merozoites as antigens as previously described (32). The endpoint IFA titer was defined as the highest serum dilution that gave detectable immunofluorescence.

Antibody avidity determination. The relative avidities of the anti-gp195 antibodies were determined by ammonium thiocyanate elution of anti-gp195 antibodies in an ELISA (26). We previously determined by an ELISA and by immunoblotting that the antigenicity of gp195 coated on plastic plates or immobilized on immunoblots was not altered after exposure to ammonium thiocyanate at concentrations as high as 8 M. Rabbit sera were diluted to a point on the linear portion of the ELISA titration curve. The diluted sera were allowed to react with gp195-coated plates. After washing was done to remove unbound antibodies, ammonium thiocyanate of various molar concentrations (8.0 to 0.2 M) was added to the wells for 15 min. After seven successive washings were done, the bound antibodies were detected by incubation with a goat antirabbit immunoglobulin G-peroxidase conjugate as described above. The molar concentration of thiocyanate ion required to elute 50% of the bound antibodies (avidity index) was used to compare the relative avidities of the rabbit antibodies.

In vitro parasite growth inhibition assay. The ability of rabbit anti-gp195 sera from different adjuvant groups to inhibit parasite growth in vitro was examined with an in vitro assay as previously described (14). Serum samples (preimmune sera) collected from each rabbit prior to immunization were used as controls. For some experiments, rabbit antibodies were isolated by protein G affinity chromatography. The bound antibodies were eluted with 0.1 M glycine (pH 2.5), neutralized with 1 M Tris (pH 8.0), and dialyzed against RPMI 1640 medium. Parasite cultures were synchronized by

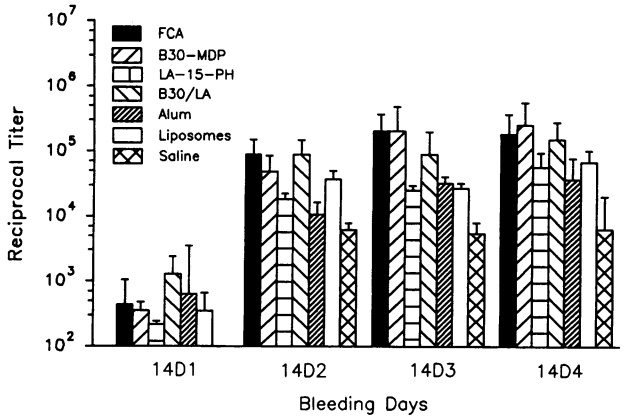

FIG. 1. gp195-specific ELISA titers of sera from rabbits immunized with gp195 and different adjuvant formulations. Sera were obtained 14 days after the primary (14D1), secondary (14D2), tertiary (14D3), and quarternary (14D4) immunizations. Endpoint tiers are defined as serum dilutions having an optical density of 0.2 and are expressed as the geometric mean ± standard deviation. The antibody titers of the FCA, B30-MDP, and B30-MDP–LA-15-PH (B30/LA) formulations were significantly higher than were those of the other adjuvant formulations ($P < 0.05$, one-tailed t test) for the 14D2, 14D3, and 14D4 bleedings. Titer differences among the FCA, B30-MDP, and B30-MDP–LA-15-PH formulations were not significant.

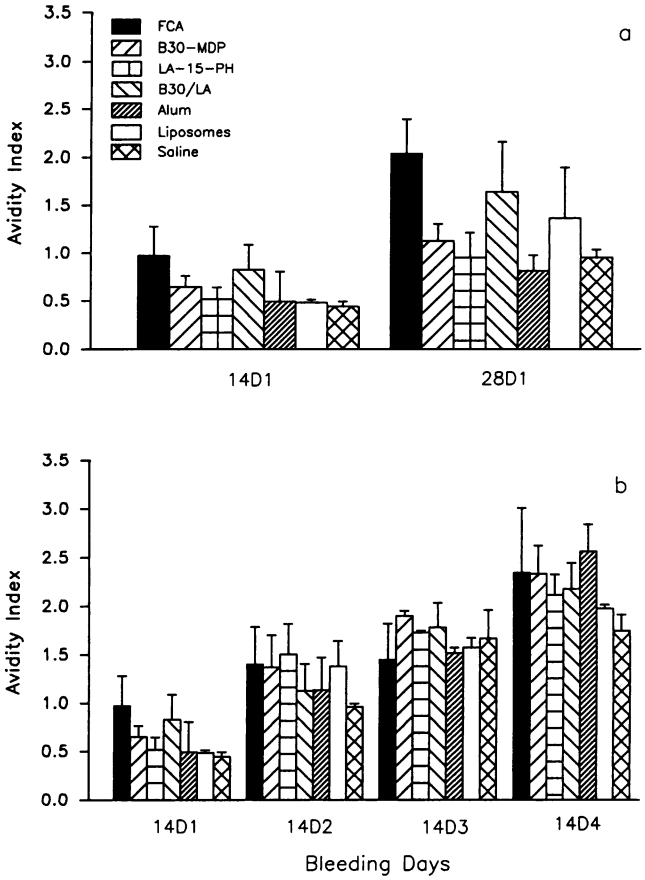

FIG. 2. Relative avidities of rabbit anti-gp195 antibodies as determined by ammonium thiocyanate elution (see the text). The avidity index is expressed as the molar concentration of ammonium thiocyanate ion required to elute 50% of the bound anti-gp195 antibodies in an ELISA. (a) Relative avidities of rabbit antibodies obtained 14 days (14D1) and 28 days (28D1) after the primary immunization. Values for FCA and B30-MDP–LA-15-PH (B30/LA) were significantly higher than those for the others ($P < 0.05$, one-tailed t test). (b) Relative avidities of rabbit antibodies obtained 14 days after the primary (14D1), secondary (14D2), tertiary (14D3), and quarternary (14D4) immunizations. The data shown are the geometric mean ± standard deviation.

sorbitol lysis to select for late trophozoite and schizont stages. Infected erythrocytes were adjusted to a parasitemia of approximately 0.5% and a hematocrit of 0.8%. Rabbit preimmune or immune sera were added to a final concentration of 15%, and 200 μl of the culture suspension was added in duplicate wells to a 96-well microtiter plate. Cultures were incubated for 72 h, and the parasitemia was determined by microscopy. Inhibition assays were performed twice, and the results represent the averages of two separate experiments.

Datum analysis. Comparisons among adjuvant formulations were made with the geometric mean values of each formulation. The data were log transformed, and statistical analyses were performed with the Student t test and the Pearson correlation analysis.

RESULTS

Anti-gp195 antibody responses. Serum antibodies of rabbits immunized with gp195 in combination with various adjuvants were assayed for anti-gp195 antibody titers by an ELISA with purified gp195 as the antigen. Figure 1 shows the ELISA titers of rabbit sera taken 14 days after each immunization. Primary antibody responses were detected in most adjuvant formulations; however, the ELISA titers were low (<1/2,000). After boosting, high titers of anti-gp195 antibodies were detected by the ELISA in several adjuvant formulations. Animals immunized with gp195 in FCA, B30-MDP–liposomes, and B30-MDP–LA-15-PH–liposomes consistently produced higher antibody titers than did those immunized with gp195 in other adjuvant formulations ($P < 0.05$, one-tailed t test), with peak titers of 1/254,000 ± 1/159,000, 1/303,000 ± 1/176,000, and 1/177,000 ± 1/107,000, respectively. There were no significant differences in titers among these three adjuvant formulations. Rabbits receiving gp195 in liposomes and with alum had lower levels (1/72,000 ± 1/33,000 and 1/44,000 ± 1/32,000, respectively) of anti-gp195 antibodies. gp195 in LA-15-PH–liposomes induced antibody levels similar to those induced when alum and liposomes were used. gp195 in saline was weakly immunogenic (<1/10,000). The ability of different adjuvants to induce antibodies reactive with native gp195 on mature schizonts or merozoites was investigated by an IFA with acetone-fixed infected erythrocytes as the antigens. All rabbit sera had antibodies that reacted with gp195 on merozoites. IFA reactivity was highest among animals immunized with FCA, B30-MDP, and B30-MDP–LA-15-PH, with titers of 1/27,000 ± 1/10,000, 1/115,000 ± 1/72,000, and 1/31,250, respectively, while IFA reactivity was lower with LA-15-PH (1/4,600 ± 1/2,900), alum (1/6,250), liposomes (1/14,600 ± 1/14,000), and saline (1/2,900 ± 1/2,800). Anti-gp195 antibody responses, as determined by the IFA, correlated well with ELISA titers ($r = 0.74$, $P < 0.01$).

Antibody avidity. The avidity of the anti-gp195 antibodies was examined by the ammonium thiocyanate elution method in an ELISA. Figure 2 shows the avidities of anti-gp195 antibodies from the primary through the quarternary immu-

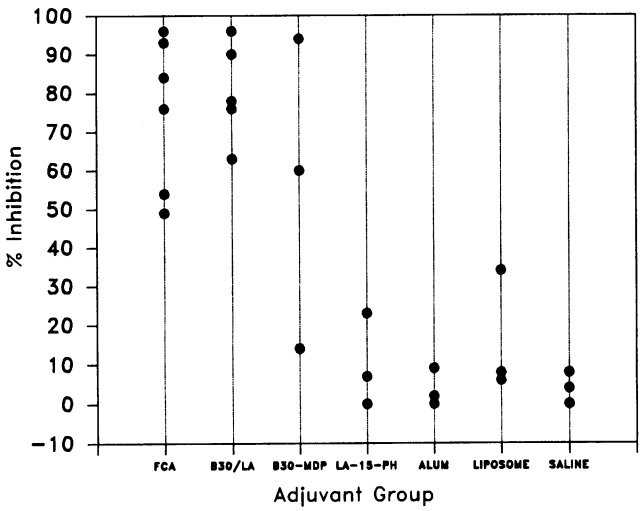

FIG. 3. In vitro parasite growth inhibition by rabbit anti-gp195 sera taken 14 days after the quarternary immunization. The degree of inhibition was determined on the basis of the percentage of inhibition of growth over a 72-h period by comparing the increase in the percent parasitemia in cultures containing the preimmune rabbit serum at 72 h (P) with the increase in the percent parasitemia from 0 h in cultures containing the respective immune rabbit serum at 72 h (T), according to the following formula (14): $100 \times [(P - 0\text{ h}) - (T - 0\text{ h})/(P - 0\text{ h})]$. Each datum point represents the mean value for each rabbit serum. B30/LA, B30-MDP–LA-15-PH.

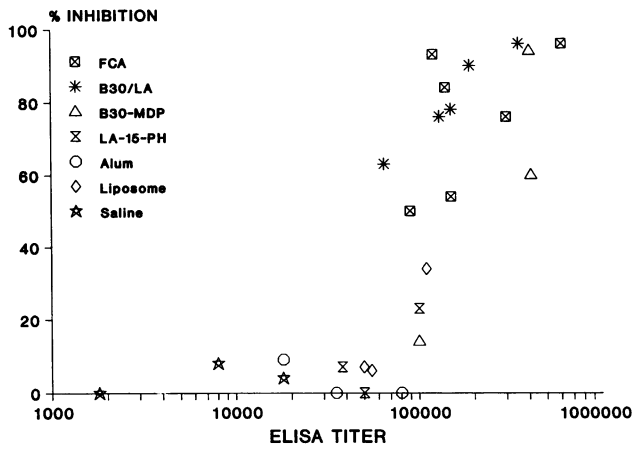

FIG. 4. Correlation between antibody responses to gp195 and the degree of parasite inhibition by sera from rabbits immunized with gp195 in different adjuvants. Each datum point represents the antibody titer and serum inhibitory activity of an individual rabbit. Sera were obtained 14 days after the quaternary immunization. For FCA, $r = 0.54$, not significant; for B30-MDP–LA-15-PH (B30/LA), $r = 0.92$, $P < 0.05$; for B30-MDP, $r = 0.54$, not significant; for all other adjuvants, no significant correlation.

nizations. Antibodies collected after the primary immunization showed maturation (increase in avidity indexes) of avidity between 14 and 28 days after immunization (Fig. 2a). The relative avidities differed among the adjuvant formulations during the primary immunization. Antibodies induced by FCA and B30-MDP–LA-15-PH–liposomes had higher avidities than did those induced by B30-MDP–liposomes, LA-15-PH–liposomes, alum, and saline ($P < 0.05$, one-tailed t test). With subsequent booster injections, the relative avidities of all adjuvant formulations increased and differences among adjuvant formulations were no longer apparent. In general, there was a slight increase in avidity with each immunization. There was no correlation between ELISA or IFA titers and the avidities of the anti-gp195 antibodies.

In vitro inhibitory activities of rabbit anti-gp195 sera. The biological activity of the anti-gp195 antibodies induced by the adjuvants was examined by an in vitro parasite growth inhibition assay. Figure 3 shows the percent growth inhibition of each rabbit anti-gp195 serum (14 days after the quaternary immunization) for different adjuvant formulations. Serum samples from the six rabbits immunized with gp195-FCA had inhibitory activities ranging from 50 to 96% (average, 75.3% ± 19%). Immunoglobulin G purified from pooled sera from these rabbits inhibited parasite growth by 80% ± 1% at a concentration of 750 μg/ml. B30-MDP–LA-15-PH–liposomes induced anti-gp195 antibodies that inhibited parasite growth by 60 to 96% (average, 80.6% ± 13%). B30-MDP alone produced less consistent inhibition. Serum samples from two of three rabbits inhibited parasite growth by 60 and 90%, while a serum sample from the third rabbit had low (14%) inhibitory activity. LA-15-PH–liposomes did not induce significant levels of inhibitory activity (average, 10% ± 11%). Similarly, little or no inhibition was observed for sera from rabbits immunized with gp195 in alum, liposomes alone, or saline.

The relationship between the anti-gp195 antibody titer (ELISA) and the degree of parasite growth inhibition is illustrated in Fig. 4. All rabbit sera with inhibitory activities of ≥50% had anti-gp195 antibody titers of ≥1/70,000. However, not all sera with anti-gp195 antibody titers of greater than 1/70,000 had inhibitory activities of greater than 50%. For example, all sera from rabbits receiving the FCA and B30-MDP–LA-15-PH formulations and sera from two of three rabbits receiving the B30-MDP formulation had ELISA titers of ≥1/70,000 and inhibited growth by ≥50%. On the other hand, sera from one rabbit each from the groups receiving the alum, liposome, LA-15-PH, and B30-MDP adjuvant formulations had ELISA titers of ≥1/70,000 but inhibited growth by ≤50%. When rabbit sera were chosen on the basis of having an inhibitory activity of ≥50% or an ELISA titer of ≥1/70,000, there was a correlation between the ELISA titer and the degree of parasite growth inhibition ($r = 0.54$, $P < 0.05$). When these sera were analyzed separately according to adjuvant formulation, the B30-MDP–LA-15-PH–liposome formulation showed a much more linear correlation between the degree of parasite growth inhibition and the ELISA titer ($r = 0.92$, $P < 0.05$). In contrast, although all rabbits immunized with gp195-FCA had inhibitory antibodies (>50% inhibition), the correlation between the degree of parasite growth inhibition and the ELISA titer was not significant ($r = 0.54$, $P = 0.14$ [not significant]). Similarly, no significant correlation was observed for the other adjuvant formulations, although it is recognized that the numbers of animals in these groups were small.

DISCUSSION

Results from our immunization experiments indicated that gp195 incorporated in B30-MDP–liposomes or B30-MDP–LA-15-PH–liposomes consistently induced high titers of anti-gp195 antibodies. These adjuvant formulations induced higher antibody responses than did alum, and the levels achieved were comparable to those achieved with FCA. Synthetic lipid A (LA-15-PH) alone in liposomes was less

effective. Moreover, we did not see an additive effect of LA-15-PH-liposomes over that of gp195-liposomes. Our results for MPL in rabbits contrast with those described by Mashihi et al. (20) and Tsujimoto et al. (38), who showed that MPL or its derivatives enhanced antibody responses to influenza subunit vaccine and hepatitis B vaccine in mice over the levels obtained with alum or liposomes. Richards et al. (27) studied the antibody responses to the recombinant CSP protein ($R32tet_{32}$) of *P. falciparum* in rabbits and monkeys. They showed that immunization with alum-adsorbed liposomes containing MPL and $R32tet_{32}$ produced higher antibody responses than did that with alum and liposomes alone. One explanation for the differences in the results could be differences in animal species and the nature of the antigens used. We have conducted a preliminary immunogenicity study of gp195 in mice with the same gp195 and immunomodulators described here (12). LA-15-PH–liposomes or B30-MDP–LA-15-PH–liposomes induced higher gp195 antibody responses than did FCA in mice. Moreover, unlike the situation in rabbits, this synthetic lipid A compound was far superior to alum in inducing anti-gp195 antibodies in mice (12). Thus, it appears that the adjuvant effect of lipid A is species dependent.

Antibody affinity or avidity is an important factor in various immunological and biological reactions, and high-affinity antibodies have been shown to be more desirable than low-affinity antibodies in many biological reactions (35). Therefore, antibody responses to gp195 were also compared in terms of avidity. Both FCA and B30-MDP–LA-15-PH produced significantly more high-avidity antibodies than did most other adjuvant formulations during the primary immunization. However, after subsequent boosting, differences in avidity among the formulations were not significant. This result suggests that successively more high-affinity antibodies were selected regardless of the adjuvant used. Since gp195 is a large and complex protein that carries a substantial number of B cell epitopes, our antibody assay reflects the overall antibody avidity for the complete molecule. Therefore, differences in the avidities of antibodies for specific epitopes may be masked. Lew et al. immunized rabbits with a synthetic octapeptide corresponding to the 3′ repeat sequence (EENVEHDA) of the *P. falciparum* ring-infected erythrocyte surface antigen, with FCA, MDP, and alum as adjuvants (17). The avidities of these peptide-specific antibodies, as measured by ammonium thiocyanate elution, differed among the adjuvant formulations. The differences in avidity persisted throughout the course of the study. Perhaps such differences in antibody avidity would be apparent if the gp195 responses were analyzed with respect to the recognition of individual epitopes. Our study also showed that antibody titer and avidity varied independently of each other, in agreement with the results of previous studies on other vaccine systems (17, 26).

We previously showed that *Aotus* monkeys immunized with gp195–FCA were protected from lethal *P. falciparum* infections (33). Prechallenge sera from protected monkeys inhibited parasite growth in vitro (>90%), while sera from unprotected monkeys had no effect (14). Therefore, the ability of anti-gp195 sera or antibodies to inhibit parasite growth in vitro correlates with gp195 vaccine-induced immunity. Immune sera and purified immunoglobulin G from rabbits immunized with gp195–FCA were also inhibitory (14). In the present study, anti-gp195 sera induced by two synthetic adjuvant formulations, namely, B30-MDP–LA-15-PH–liposomes and B30-MDP–liposomes, inhibited parasite growth to a similar extent as did FCA. The results provide indirect evidence that these formulations can potentiate protective immunity specific for gp195. An analysis of antibody titer and the degree of inhibition suggested that a minimum level (≥1/70,000) of anti-gp195 antibodies may be needed to exert a significant biological effect in vitro. However, the antibody titer may not be the only factor influencing in vitro inhibitory activity, as suggested by the following observations. First, sera from several rabbits had titers of ≥1/70,000 but were not inhibitory. Second, a strong correlation between the ELISA titer and in vitro inhibition was observed in the B30-MDP–LA-15-PH-immunized rabbits but not the FCA-immunized rabbits. Finally, in a previous vaccination study of *Aotus* monkeys with purified gp195 and rhoptry proteins as vaccine antigens, we obtained protection with FCA as the adjuvant, while the same antigens failed to confer protection when used with another synthetic MDP derivative, MDP-Lys(L18), despite the fact that the antibody titers were similar to those in the FCA-immunized monkeys (32). In addition, only the serum samples from the FCA-immunized monkey strongly inhibited parasite growth in vitro (14). These observations, together with those of the present study, suggest that protective immunity against gp195 may depend in part on the quality of the antibody response, which in turn may be affected by different adjuvants. One explanation may be that the specificities of the anti-gp195 antibody responses induced by different adjuvants are different and that, of the total number of B cell epitopes on gp195, only a limited number are involved in parasite inhibition and/or immunity. FCA may induce more antibodies against a greater number of "irrelevant" B cell epitopes in addition to the inhibitory epitope(s) on gp195 than does the B30-MDP–LA-15-PH–liposome formulation, possibly resulting in a poorer correlation between the overall gp195 titer and the degree of in vitro inhibition for FCA than for the B30-MDP–LA-15-PH-liposome formulation. Therefore, although LA-15-PH alone was not efficacious in stimulating inhibitory antibodies, it may help to induce antibodies required for inhibition, since B30-MDP alone was less consistent in stimulating inhibitory antibodies. Along the same lines, gp195 given with other adjuvants may have failed to elicit the antibodies needed for in vitro parasite inhibition or in vivo protection, despite high antibody titers. Studies are in progress to compare the specificities of the anti-gp195 antibodies in rabbits.

Our studies provide encouraging evidence that adjuvant formulations based on low-toxicity synthetic B30-MDP alone or in combination with LA-15-PH can replace FCA in a gp195-based malaria vaccine. The ability of these formulations to elicit gp195-specific protective immunity against *P. falciparum* in the *Aotus* monkey model should now be determined. Once the protective efficacy of these adjuvants has been demonstrated in vivo, they may replace FCA as a safe and effective adjuvant for the evaluation of the immunogenicity of gp195-based recombinant or synthetic vaccines. In addition, the effectiveness of these adjuvants in inducing protective immunity against other malarial antigens, such as the CSP protein, also merits investigation. Finally, our studies have concentrated on the humoral response to gp195. Further experiments will focus on evaluating the potential of these immunomodulators to elicit different components of the cellular immune response to gp195.

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REFERENCES

1. **Alving, C. R., R. L. Richards, J. Moss, L. I. Alving, J. D. Clements, T. Shiba, S. Kotani, R. A. Wirtz, and W. T. Hockmeyer.** 1986. Effectiveness of liposomes as potential carriers of vaccines. Application to cholera toxin and human malaria sporozoite antigen. Vaccine **4**:166–172.
2. **Alving, C. R., S. Shichijo, and I. Mattsby-Baltzer.** 1984. Preparation of liposomes in immunological studies, p. 158–175. *In* G. Gregoriadis (ed.), Liposome technology, vol. 2. CRC Press, Inc., Boca Raton, Fla.
3. **Aprile, M. A., and A. C. Wardlaw.** 1966. Aluminium compounds as adjuvants for vaccine and toxoids in man: a review. Can. J. Public Health **57**:343–354.
4. **Audibert, F. M., G. Przewlocki, C. D. Leclerc, M. E. Jolivet, H. S. Gras-Masse, A. L. Tartar, and L. A. Chedid.** 1984. Enhancement by murabutide of the immune response to natural and synthetic hepatitis B surface antigens. Infect. Immun. **45**:261–266.
5. **Ballou, W. R., J. A. Sherwood, F. A. Neva, D. M. Gordon, R. A. Wirth, G. F. Wasserman, C. L. Diggs, S. L. Hoffman, M. R. Hollingdale, W. T. Hockmeyer, I. Scheider, J. F. Young, P. Reeve, and J. D. Chulay.** 1987. Safety and efficacy of a recombinant DNA *Plasmodium falciparum* sporozoite vaccine. Lancet **i**:1277–1281.
6. **Bomford, R.** 1980. The comparative selectivity of adjuvants for humoral and cell-mediated immunity. II. Effect of delayed-type hypersensitivity in the mouse and guinea pig, and cell-mediated immunity to tumour antigens in the mouse of Freund's incomplete and complete adjuvants, alhydrogel, *Corynebacterium parvum*, *Bordetella pertussis*, muramyl dipeptide and saponin. Clin. Exp. Immunol. **39**:435–441.
7. **Chang, S. P., G. S. N. Hui, A. Kato, and W. A. Siddiqui.** 1989. Generalized immunological recognition of the major merozoite surface antigen (gp195) of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA **86**:6343–6347.
8. **Clough, E. R., F. M. Audibert, J. W. Barnwell, D. H. Schlesinger, R. Arnon, and L. A. Chedid.** 1985. Biologically active antibodies elicited by a synthetic circumsporozoite peptide of *Plasmodium knowlesi* administered in saline with muramyl dipeptide derivative. Infect. Immun. **48**:839–841.
9. **Dijkstra, J., J. W. Mellors, J. L. Ryan, and F. C. Szoka.** 1987. Modulation of the biological activity of bacterial endotoxin by incorporation into liposomes. J. Immunol. **138**:2663–2670.
10. **Hall, R., J. E. Hyde, M. Goman, D. L. Simmons, I. A. Hope, M. Mackay, J. Scaife, B. Merkli, R. Richle, and J. Stocker.** 1984. Major surface antigen of a human malaria parasite cloned and expressed in bacteria. Nature (London) **311**:379–382.
11. **Herrington, D. A., D. F. Clyde, G. Losonsky, M. Cortesia, J. R. Murphy, J. Davis, S. Baqar, A. M. Felix, E. P. Heimer, D. Gillessen, E. Nardin, R. S. Nussenzweig, V. Nussenzweig, M. R. Hollingdale, and M. M. Levine.** 1987. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. Nature (London) **328**:257–259.
12. **Hui, G. S. N., S. P. Chang, L. Q. Tam, A. Kato, S. E. Case, C. Hashiro, S. Kotani, T. Shiba, S. Kusumoto, and W. A. Siddiqui.** 1990. Characterization of antibody responses induced by different synthetic adjuvants to the *Plasmodium falciparum* major merozoite surface precursor protein, gp195, p. 477–484. *In* F. Brown, R. M. Chanock, H. S. Ginsberg, and R. A. Lerner (ed.), Modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Hui, G. S. N., K. L. Palmer, and W. A. Siddiqui.** 1984. Use of plasma for continuous in vitro cultivation of *Plasmodium falciparum*. Trans. R. Soc. Trop. Med. Hyg. **78**:625–626.
14. **Hui, G. S. N., and W. A. Siddiqui.** 1987. Serum from Pf195 protected *Aotus* monkeys inhibits *Plasmodium falciparum* growth in vitro. Exp. Parasitol. **64**:519–522.
15. **Kotani, S., H. Takada, M. Tsujimoto, T. Ogawa, I. Takahashi, T. Ikeda, K. Otsuka, H. Shimauchi, N. Kasai, J. Mashimo, S. Nagao, A. Tanaka, S. Tanaka, K. Harada, K. Nagaki, H. Kitamura, T. Shiba, S. Kusumoto, M. Imoto, and H. Yoshimura.** 1985. Synthetic lipid A with endotoxic and related biological activities comparable to those of a natural lipid A from *Escherichia coli* Re-mutant. Infect. Immun. **49**:225–237.
16. **Lambros, C., and J. Vanderberg.** 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. **65**:418–420.
17. **Lew, A. M., R. F. Anders, S. J. Edwards, and C. J. Langford.** 1988. Comparison of antibody avidity and titer elicited by peptide as a protein conjugate or as expressed in vaccinia. Immunology **65**:311–314.
18. **Lise, D., C. Dubeaux, D. Tello, D. Mazier, M. Jolivet, D. H. Schlesinger, F. Audibert, and L. Chedid.** 1988. Construction of immunogens for synthetic malaria vaccines. Biochem. Biophys. Res. Commun. **153**:31–38.
19. **Marx, P. A., N. C. Pedersen, N. W. Lerche, K. G. Osborn, L. J. Lowenstine, A. A. Lackner, D. H. Maul, H. S. Kwang, J. D. Kluge, C. P. Zaiss, V. Sharpe, A. P. Spinner, A. C. Allison, and M. B. Gardner.** 1986. Prevention of simian acquired immune deficiency syndrome with a Formalin-inactivated type D retrovirus vaccine. J. Virol. **60**:431–435.
20. **Mashihi, K. N., W. Lange, W. Brehmer, and E. Ribi.** 1986. Immunopharmacological activities of non-toxic lipid A: enhancement of non-specific resistance in combination with trehalose dimycolate against viral infection and adjuvant effects. Int. J. Immunopharmacol. **8**:339–347.
21. **Morgan, A. J., M. A. Epstein, and J. R. North.** 1984. Comparative immunogenicity studies on Epstein-Barr virus membrane antigen (MA) gp340 with novel adjuvants in mice, rabbits, and cotton-top tamarins. J. Med. Virol. **13**:281–292.
22. **Naylor, P. T., H. S. Larsen, L. Huang, and B. T. Rouse.** 1982. In vivo induction of anti-herpes simplex virus immune response by type 1 antigens and lipid A incorporated into liposomes. Infect. Immun. **36**:1209–1216.
23. **Patarroyo, M. E., R. Amador, P. Clavijo, A. Moreno, F. Guzman, P. Romero, R. Tascon, A. Franco, L. A. Murillo, G. Ponton, and G. Trujillo.** 1988. A synthetic vaccine protects humans against challenge with asexual blood stages of *Plasmodium falciparum* malaria. Nature (London) **332**:158–161.
24. **Perrin, L. H., B. Merkli, M. Loche, C. Chizzolini, J. Smart, and R. Richle.** 1985. Antimalaria immunity in *Saimiri* monkeys: immunization with surface components of asexual blood stages. J. Exp. Med. **160**:441–451.
25. **Potter, C. W., R. Jennings, J. P. Phair, A. Clarke, and C. H. Stuart-Harris.** 1977. Dose-response relationship after immunization of volunteers with a new, surface-antigen-adsorbed influenza virus vaccine. J. Infect. Dis. **135**:423–431.
26. **Pullen, G. R., M. G. Fitzgerald, and C. S. Hosking.** 1986. Antibody avidity determination by ELISA using thiocyanate elution. J. Immunol. Methods **86**:83–87.
27. **Richards, R. L., M. D. Hayre, W. T. Hockmeyer, and C. R. Alving.** 1988. Liposomes, lipid A, and aluminum hydroxide enhance the immune response to a synthetic malaria sporozoite antigen. Infect. Immun. **56**:682–686.
28. **Richardson, E. C., B. Banerji, R. C. Seid, Jr., J. Levin, and C. R. Alving.** 1983. Interaction of lipid A and liposome-associated lipid A with *Limulus polyhemus* amoebocytes. Infect. Immun. **39**:1385–1391.
29. **Robey, W. G., L. O. Arthur, T. J. Matthews, A. Langlois, T. D. Copeland, N. W. Lerche, S. Oroszlan, D. P. Bolognes, R. V. Gilden, and P. J. Fischinger.** 1986. Prospect for prevention of human immunodeficiency virus infection: purified 120-kDa envelope glycoprotein induces neutralizing antibody. Proc. Natl. Acad. Sci. USA **83**:7023–7027.
30. **Sanchez-Pescador, L., R. L. Burke, G. Ott, and G. Van Nest.** 1988. The effect of adjuvants on the efficacy of a recombinant herpes simplex virus glycoprotein vaccine. J. Immunol. **141**: 1720–1727.
31. **Scolnick, E. M., A. A. McLean, D. J. West, W. J. McAleer, W. J. Miller, and E. B. Buynak.** 1984. Clinical evaluation in

healthy adults of a hepatitis B vaccine made by recombinant DNA. JAMA **251**:2812–2815.

32. **Siddiqui, W. A., L. Q. Tam, S. C. Kan, K. J. Kramer, S. E. Case, K. L. Palmer, K. M. Yamaga, and G. S. N. Hui.** 1986. Induction of protective immunity to monoclonal-antibody-defined *Plasmodium falciparum* antigens requires strong adjuvants in *Aotus* monkeys. Infect. Immun. **52**:314–318.
33. **Siddiqui, W. A., L. Q. Tam, K. J. Kramer, G. S. N. Hui, S. E. Case, K. M. Yamaga, S. P. Chang, E. B. T. Chan, and S. C. Kan.** 1987. Merozoite surface coat precursor protein completely protects *Aotus* monkeys against *Plasmodium falciparum* malaria. Proc. Natl. Acad. Sci. USA **84**:3014–3018.
34. **Siddiqui, W. A., D. W. Taylor, S. C. Kan, K. J. Kramer, S. M. Richmond-Crum, S. Kotani, T. Shiba, and S. Kusumoto.** 1978. Vaccination of experimental monkeys against *Plasmodium falciparum*: a possible safe adjuvant. Science **201**:1237–1239.
35. **Steward, M. W., and J. Steensgard.** 1983. Antibody affinity: thermodynamic aspects and biological significance. CRC press, Inc., Boca Raton, Fla.

35a. **Tam, L. Q.** Unpublished data.

36. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193**:673–675.
37. **Tsujimoto, M., S. Kotani, F. Kinoshita, S. Kanoh, T. Shiba, and S. Kusumoto.** 1986. Adjuvant activity of 6-*O*-acyl-muramyldipeptides to enhance primary cellular and humoral immune responses in guinea pigs: adaptability to various vehicles and pyrogenicity. Infect. Immun. **53**:511–516.
38. **Tsujimoto, M., S. Kotani, T. Okunaga, T. Kubo, H. Takada, T. Kubo, T. Shiba, S. Kusumoto, T. Takahashi, Y. Goto, and F. Kinoshita.** 1989. Enhancement of humoral immune responses against viral vaccines by a non-pyrogenic 6-o-acyl-muramyldipeptide and synthetic low toxicity analogs of lipid A. Vaccine **7**:39–48.
39. **Tsujimoto, M., S. Kotani, T. Shiba, and S. Kusumoto.** 1986. Adjuvant activity of 6-*O*-acyl-muramyldipeptides to enhance primary cellular and humoral immune responses in guinea pigs: dose-response and local reactions observed with selected compounds. Infect. Immun. **53**:517–521.
40. **Warren, H. S., F. R. Vogel, and L. A. Chedid.** 1986. Current status of immunological adjuvants. Annu. Rev. Immunol. **4**:369–388.