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Liposomes, Lipid A, and Aluminum Hydroxide Enhance the Immune Response to a Synthetic Malaria Sporozoite Antigen

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A liposome-encapsulated cloned protein ($R32tet_{32}$) containing sequences from the tetrapeptide repeat region of the circumsporozoite protein of *Plasmodium falciparum* sporozoites was examined for immunogenicity with rabbits and monkeys. Effects of adjuvants were tested by encapsulation of the antigen in liposomes either lacking or containing lipid A and adsorption with aluminum hydroxide (ALUM). When rabbits were immunized with $R32tet_{32}$ alone, a primary antibody response was not seen and a secondary response did not appear until 32 to 36 weeks after boosting. Immunization with ALUM-adsorbed $R32tet_{32}$ resulted in a minimal primary antibody response. A moderate secondary antibody response was detected within 2 weeks after boosting, but antibody levels decreased to preimmunization levels 8 weeks after boosting. When $R32tet_{32}$ was encapsulated in liposomes containing lipid A, strong primary and secondary antibody responses were observed. Strong primary and secondary responses also were obtained when $R32tet_{32}$ was encapsulated in liposomes either containing or lacking lipid A and the liposomes were adsorbed with ALUM. The strongest antibody response was obtained by immunization with ALUM-adsorbed liposomes containing lipid A and $R32tet_{32}$, suggesting that the adjuvant effects of liposomes, lipid A, and ALUM were additive or synergistic.

Recent advances in malaria research have resulted in identification of the complete structure of the gene encoding the circumsporozoite (CS) protein on the surface of the sporozoite of the human malaria parasite *Plasmodium falciparum* (10). This development has provided an opportunity for the use of synthetic antigens as the basis for vaccines against malaria. Toward this goal, two approaches are being pursued, namely, development of synthetic peptide-carrier protein conjugates as antigens (7, 31) and development of cloned synthetic proteins as antigens (17, 30). It is known that immunization with killed sporozoites, or with peptides derived from the repeat region of the CS protein, or with a cloned protein that contains sequences from the repeat region of the CS protein, induces antibodies reactive with intact sporozoites or CS protein (8, 13, 19, 21, 31). Three clinical trials have demonstrated that killed sporozoites (8), a cloned protein (6), or a peptide-toxoid conjugate (15) can induce protective immunity to *P. falciparum* in humans.

There is a widespread belief that optimal methods for immunization against certain synthetic antigens may require the use of adjuvants, and this has stimulated a considerable amount of research aimed at developing new or improved adjuvants (12). The most widely used adjuvants consist of aluminum compounds, particularly aluminum hydroxide (ALUM), which is used in diphtheria and tetanus toxoid vaccines (12). Current adjuvants that have shown promise include, among others, a variety of lipophilic derivatives of muramyl dipeptide (25, 28) and liposomes or combinations of liposomes with lipophilic muramyl dipeptide or lipid A (reviewed in reference 1). One of the peptides tested for immunogenicity after encapsulation into liposomes was a polypeptide derived from hepatitis B virus. When compared with ALUM-adsorbed polypeptide derived from hepatitis B virus, liposome-encapsulated polypeptide induced a higher antipeptide immune response (24). We have found previously that an antigen consisting of tetrapeptide repeat sequences from the CS protein conjugated to bovine serum albumin is nonimmunogenic by itself but is highly immunogenic in liposomes and more immunogenic yet in liposomes containing lipid A (4).

Previous work in our program has shown that a cloned protein ($R32tet_{32}$) that contains sequences from the repeat region of the CS protein of *P. falciparum* is more immunogenic when combined with ALUM (30). The purpose of this study was to evaluate ALUM, liposomes, and liposomes containing lipid A as potential adjuvants for stimulating immunity against $R32tet_{32}$.

MATERIAL AND METHODS

Materials. Lipids were obtained from the following sources: dimyristoyl phosphatidylcholine from Sigma Chemical Co., St. Louis, Mo., or Avanti Polar Lipids, Inc., Birmingham, Ala.; cholesterol from Calbiochem-Behring, La Jolla, Calif.; dicetyl phosphate from K & K Laboratories, Inc., Plainview, N.Y.; and dimyristoyl phosphatidylglycerol from Avanti. Lipid A was prepared as described previously (5) from *Escherichia coli* O111 lipopolysaccharide (Difco Laboratories, Detroit, Mich.). Aluminum hydroxide gel (Amphojel; Wyeth Laboratories, Philadelphia, Pa.) was used as the ALUM source.

A synthetic protein ($R32tet_{32}$) containing 32 tetrapeptide repeats from the CS protein of *P. falciparum* and 32 amino acids derived from the tetracycline resistance gene of the cloning vector was used as the antigen in the studies reported here (29, 30). This is the antigen that was used in a recent clinical trial (6).

Preparation of liposomes. Liposomes were prepared essentially as described previously (5). Liposomes for the dose-response experiment (see Fig. 2) and for the primary immunization for all but one of the other rabbit experiments (see Fig. 1, 4, and 5) were composed of dimyristoyl phosphatidylcholine, cholesterol, and dicetyl phosphate in mole ratios of

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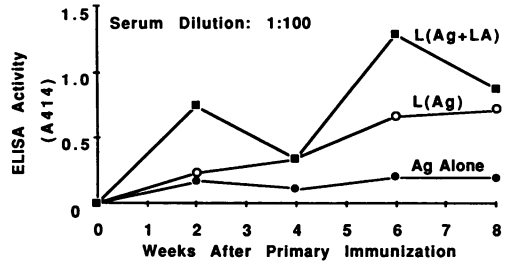

FIG. 1. Enhancement of antibody activity by encapsulation of $R32tet_{32}$ in liposomes lacking or containing lipid A. Each point represents the mean ELISA antibody response (four rabbits per group) after subtraction of the preimmune value at a serum dilution of 1:100. Rabbits were immunized at 0 and 4 weeks, with each animal receiving 2 μg of $R32tet_{32}$ per injection. Ag Alone, free $R32tet_{32}$; L(Ag), $R32tet_{32}$ encapsulated in liposomes lacking lipid A; L(Ag + LA), $R32tet_{32}$ encapsulated in liposomes containing lipid A.

1.0:0.75:0.11. All other liposomes were composed of dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, and cholesterol in mole ratios of 0.9:0.1:0.75. Lipid A was included in the liposomes where indicated at 20 nmol of lipid A phosphate per μmol of phospholipid. Lipids were dried under vacuum from chloroform solution in pear-shaped flasks. After addition of a small quantity of acid-washed 0.5-mm glass beads, the liposomes were swollen in solutions of $R32tet_{32}$ diluted in 0.15 M NaCl by 2 min of vigorous shaking in a vortex mixer. Unencapsulated antigen was removed by centrifugation at 12,000 to 15,000 × *g* for 10 min at 20°C, and the liposomal pellets were then washed in 0.15 M NaCl by centrifugation as described above. The washed liposomes were suspended in 0.15 M NaCl at a total phospholipid concentration of 10 mM for rabbit immunizations and 20 mM for monkey immunizations.

Immunization procedures. Rabbits were immunized with one of the following: $R32tet_{32}$ alone, $R32tet_{32}$ adsorbed with ALUM, $R32tet_{32}$ encapsulated in liposomes containing or lacking lipid A, or $R32tet_{32}$ encapsulated in liposomes containing or lacking lipid A and then adsorbed with ALUM. The ALUM adsorbates were prepared as described previously (30) except that the antigen dose was approximately 2 μg and the ALUM was diluted in 0.15 M NaCl.

For immunizations with ALUM-immunogen mixtures, rabbits were injected intramuscularly with 0.5 ml of an inoculum consisting of immunogen mixed with ALUM. ALUM adsorbates of liposome-encapsulated antigen contained an amount of liposomes containing 10 μmol of dimyristoyl phosphatidylcholine in each 0.5-ml dose. For all other antigen preparations, each rabbit received 1-ml doses. For the $R32tet_{32}$ dose-response experiment (see Fig. 2), both injections were given intravenously in the ear vein. For all other immunizations, the primary injection was given intravenously and the boosting injection was given intramuscularly.

Rhesus monkeys were immunized at 0 and 4 weeks with 40-μg doses of $R32tet_{32}$ either as free antigen, ALUM adsorbate, or ALUM adsorbate of $R32tet_{32}$ encapsulated in liposomes containing lipid A. All injections were done intramuscularly, and each dose was in a volume of 0.5 ml.

ELISA. Enzyme-linked immunosorbent assays (ELISA) were carried out as described previously (30). Wells of polystyrene microtiter plates were each coated with 0.1 μg of $R32tet_{32}$ in 0.01 M phosphate-buffered saline (PBS), pH 7.4. Approximately 18 h later, the contents of the wells were aspirated, the wells were filled with blocking buffer (1.0% bovine serum albumin, 0.5% casein, 0.01% thimerosal, and 0.005% phenol red in PBS) and held for 1 h at room temperature. Rabbit sera to be tested were diluted in blocking buffer, and aliquots of each dilution were added to triplicate wells. After a 2-h incubation at room temperature, the contents of the wells were aspirated, the wells were washed three times with PBS-Tween 20, and 50 μg of horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin G (IgG) (Bio-Rad Laboratories, Richmond, Calif.) diluted 1:500 with 10% heat-inactivated human serum in PBS was added to each well. After 1 h, the contents of the wells were aspirated, the wells were washed three times with PBS-Tween 20, and 150 μl of peroxidase substrate in buffer was then added to each well. The A_{414} was determined 1 h later with an ELISA plate reader (Titertek Multiskan, Flow Laboratories, Inc., McLean, Va.).

RESULTS

Effect of encapsulation of $R32tet_{32}$ on immune response. Immunization of four rabbits with $R32tet_{32}$ encapsulated in liposomes lacking lipid A resulted in increased mean antibody levels, especially after the boost at 4 weeks (Fig. 1). This increase of the mean activity after the boost was essentially accounted for by only one of the rabbits that developed a high level of antibody activity. Antibody levels in the other three rabbits in this group did not increase above preimmunization levels after boosting. When lipid A was also included in the immunizing liposomes, both the primary and secondary responses were stimulated further (Fig. 1). Two out of the four rabbits immunized with antigen encapsulated in lipid A containing liposomes gave both a strong primary and secondary response, and one of the four rabbits gave a weak primary but strong secondary response.

The role of antigen dose in the immune response to liposome-encapsulated $R32tet_{32}$ is shown in Fig. 2 and 3. Antibodies against $R32tet_{32}$ were obtained in rabbits after immunization with two injections of 2, 0.2, 0.02, and even as little as 0.002 μg of $R32tet_{32}$ encapsulated in liposomes containing lipid A (Fig. 2). Even though two injections were given, immune responses were seen even after the first injection (Fig. 2, 2-week data). There were no clear differences between the values obtained with 2 and 0.02 μg or 0.02 and 0.002 μg, but apparent differences were found between 2 and 0.002 μg at 6 and 12 weeks. The data are consistent with a shallow dose-response curve that gave gradually

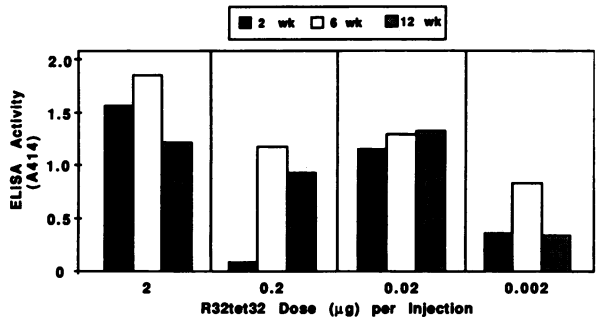

FIG. 2. Effect of antigen dose on antibody activity in rabbits immunized with $R32tet_{32}$ encapsulated in liposomes containing lipid A. Each bar represents the mean ELISA activity (two rabbits per group) after subtraction of the preimmune value at a serum dilution of 1:100. Each rabbit was injected at 0 and 4 weeks with the indicated dose of $R32tet_{32}$ encapsulated in liposomes containing lipid A.

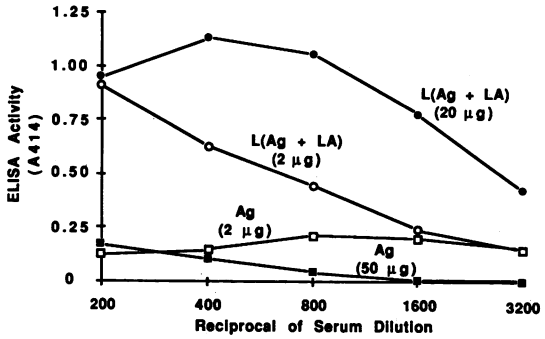

FIG. 3. ELISA antibody activity at 6 weeks in rabbits immunized twice (at 0 and 4 weeks) with 20- or 2-μg doses of $R32tet_{32}$ encapsulated in liposomes containing lipid A. Each point represents the mean ELISA antibody response (four rabbits per group) at the indicated serum dilution after subtraction of the preimmune value. For definitions of abbreviations, see the legend to Fig. 1.

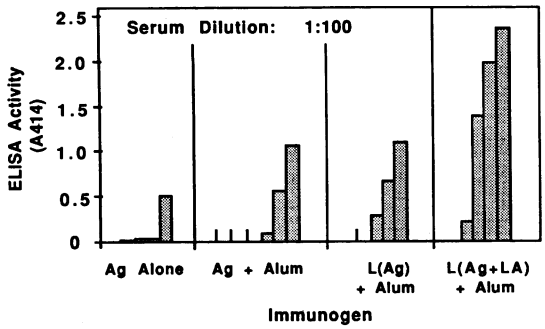

FIG. 5. ELISA antibody response of individual rabbits at 2 weeks after primary immunization. Each bar (or tick mark at the base line) represents the ELISA antibody response for a rabbit after subtraction of the preimmune activity at a serum dilution of 1:100 for six (Ag + Alum) or four (all other groups) rabbits. The abbreviations for antigen groups are the same as those shown in the legend to Fig. 4. A small vertical line (tick mark) has been placed to illustrate each animal in which no antibody activity was observed (A_{414} = 0.000 after subtraction of the preimmunization value).

diminishing immune responses that were still active with as little as 2 ng of antigen per injection. Antibody titers were higher in rabbits receiving two 20-μg doses of liposomal $R32tet_{32}$ than in rabbits receiving two 2-μg doses of liposomal $R32tet_{32}$ (Fig. 3). Much lower levels of antibody were produced in rabbits immunized with two 50-μg or two 2-μg doses of free unencapsulated $R32tet_{32}$ (Fig. 3).

Effects of ALUM on immunogenicity of free and liposomal $R32tet_{32}$. A comparison was made (Fig. 4) between 2 μg per injection of $R32tet_{32}$, ALUM-adsorbed $R32tet_{32}$, an ALUM adsorbate of $R32tet_{32}$ encapsulated in liposomes lacking lipid A, and an ALUM adsorbate of $R32tet_{32}$ encapsulated in liposomes containing lipid A. At this antigen dose, free $R32tet_{32}$ initially appeared to be nonimmunogenic, but by 36 weeks after the primary immunization, antibody activity was observed. When ALUM-adsorbed $R32tet_{32}$ was the immunogen, a short-lived secondary response was observed only at 2 to 4 weeks after the second injection (6 to 8 weeks after the primary immunization). Both of the groups immunized with liposome-ALUM combinations showed both primary and secondary antibody responses. The strongest response was obtained after immunization with [L(Ag + LA) + Alum] (Fig. 4). These data therefore show that ALUM, lipid A, and liposomes were more active when used in combination as adjuvants for the sporozoite antigen.

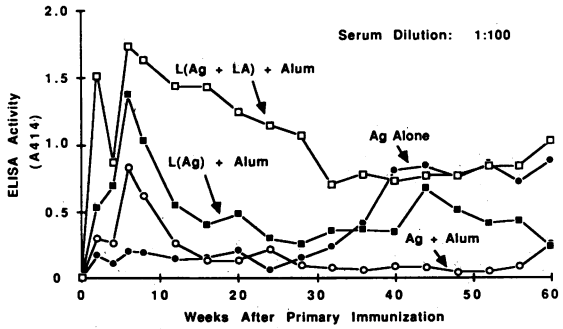

FIG. 4. Effects of ALUM, lipid A, and liposomes on mean antibody activities of rabbits injected at 0 and 4 weeks. Each point represents the mean ELISA antibody response after subtraction of the preimmune value at a serum dilution of 1:100 for six (Ag + Alum) or four (all other groups) rabbits. Ag Alone, free $R32tet_{32}$; Ag + Alum, $R32tet_{32}$ adsorbed with ALUM; L(Ag) + Alum, ALUM adsorbate of $R32tet_{32}$ encapsulated in liposomes lacking lipid A; L(Ag + LA) + Alum, ALUM adsorbate of $R32tet_{32}$ encapsulated in liposomes containing lipid A.

The data showing the primary immune response observed for each of the individual animals 2 weeks after immunization are illustrated in Fig. 5. No significant antibody activities ($A_{414} < 0.6$) were observed in the four animals that were immunized with antigen alone, and ALUM-adsorbed antigen stimulated a strong response in only one out of six rabbits immunized. In contrast, in the group injected with liposomes containing antigen and lipid A in which the liposomes were adsorbed with ALUM, three of the four animals gave a positive response, and in each case the response was higher than with any of the animals in the groups injected with antigen alone or ALUM-adsorbed antigen. Each of the four groups tested had at least one animal that responded only weakly.

The degree of antibody activity during the secondary response was strongly dependent on the time after immunization at which the serum was examined (Fig. 4). At 6 weeks after the primary immunization (2 weeks after boosting), the only group that responded poorly was the one that received $R32tet_{32}$ alone. By 28 weeks, the antibody activity had virtually disappeared from three of the four groups. Two of the four rabbits that received the ALUM adsorbate of $R32tet_{32}$ encapsulated in liposomes containing lipid A maintained a high level of antibody activity even after 28 weeks.

Immunogenicity of $R32tet_{32}$ in monkeys. Immunization of rhesus monkeys at 0 and 4 weeks with ALUM-adsorbed $R32tet_{32}$ resulted in substantial levels of antibody at 6 weeks after the primary immunization (Fig. 6). Free $R32tet_{32}$ was nonimmunogenic in monkeys over the period studied. When an ALUM adsorbate of $R32tet_{32}$ encapsulated in liposomes containing lipid A was the immunogen, high mean levels of antibody were obtained in all four of the monkeys (Fig. 6).

DISCUSSION

We have previously demonstrated that a synthetic peptide antigen consisting of an albumin-conjugated 16-amino-acid peptide sequence from the CS protein of *P. falciparum* was virtually nonimmunogenic alone but was highly immunogenic in liposomes and was even more immunogenic in liposomes containing lipid A (4). Liposomes and lipid A also have served as adjuvants for stimulating higher immune

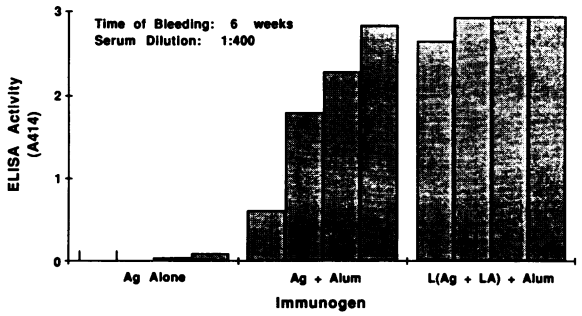

FIG. 6. Effect of ALUM, lipid A, and liposomes on ELISA antibody activity of individual rhesus monkeys. Each bar (or tick mark at the base line) represents the ELISA antibody response for a monkey (four monkeys per group) after subtraction of the preimmune value at a serum dilution of 1:400. Monkeys were immunized at 0 and 4 weeks with 40 μg of $R32tet_{32}$. Ag Alone, free $R32tet_{32}$; Ag + Alum, $R32tet_{32}$ adsorbed with ALUM; L(Ag + LA) + Alum, ALUM adsorbate of $R32tet_{32}$ encapsulated in liposomes containing lipid A. A small vertical line (tick mark) has been placed to illustrate each animal in which no antibody activity was observed (A_{414} = 0.000 after subtraction of the preimmunization value).

responses against a variety of other protein antigens, including cholera toxin (2, 4), an antigen derived from Epstein-Barr virus (14, 18), herpes simplex antigen (20), and albumin (26).

The adjuvant activity of lipid A in liposomes has been investigated for various purposes in numerous laboratories (1, 3). It has been found that liposomes can serve as a vehicle that allows expression of the adjuvant activity of lipid A, and liposomes simultaneously reduce certain unwanted side effects of lipid A. Among its other activities, lipid A causes neutropenia in rabbits, but neutropenia did not occur with liposomes containing lipid A (22). Lipid A also causes gelation in the *Limulus* lysate assay, but gelation can be abolished by incorporation of lipid A into liposomes (11, 22, 23). Lipid A-induced secretion of interleukin-1 by macrophages was also strongly reduced after incorporation of lipid A into liposomes (11).

The lipid A and liposomal lipid A used in these experiments were pyrogenic in rabbits, but pyrogenicity was reduced by incorporation of lipid A into liposomes. The relative liposomal lipid A doses required for pyrogenicity and for adjuvant activity are currently being explored. A lipid A product having markedly reduced pyrogenic activity has completed Phase I testing in humans (27), and initial results regarding its potency as an adjuvant for $R32tet_{32}$ have been quite encouraging. Among the other ingredients in the formulation used in the present study, parenterally administered liposomes per se have not yet been subjected to a formal Phase I evaluation, but studies with humans have not identified any toxic effects at therapeutically effective doses (9, 16). As mentioned earlier, ALUM is routinely used in diphtheria and tetanus toxoid vaccines (12). We do not anticipate that toxic effects will limit the potential usefulness of a liposomal sporozoite vaccine formulation.

Liposomes, lipid A, and ALUM were compared in the present study for the ability to stimulate an immune response to $R32tet_{32}$, a candidate sporozoite malaria vaccine (10, 30). This vaccine was highly immunogenic in animals (29, 30). $R32tet_{32}$ adsorbed onto ALUM was recently tested in Phase I clinical trials under an Investigational New Drug protocol as a potential vaccine against malaria, and the data showed that protective immunity could be induced in humans (6). Protection against *P. falciparum* challenge was inefficient in that only one of two volunteers that received 800 μg of antigen was protected (6). It is apparent that $R32tet_{32}$ might benefit by the use of an immunogen containing more potent adjuvant activity.

This study suggests that liposomes, lipid A, and ALUM have greater activity when used in combination for stimulating primary and secondary immune responses to $R32tet_{32}$ in rabbits. At the dose of antigen used, $R32tet_{32}$ by itself exhibited a weak primary response, and there was an 8- to 9-month delay after boosting before onset of a substantial secondary response (Fig. 4). The combination consisting of liposomes containing both antigen and lipid A, when adsorbed with ALUM, induced strong primary and long-duration secondary immune responses that were higher than those observed with any of the constituents separately or in other combinations.

Our previous study in this series was performed with a CS protein-derived synthetic peptide antigen that was virtually nonantigenic by itself in the absence of complete Freund adjuvant (4). Our success with that antigen encouraged us to examine the adjuvant effect of liposomes and lipid A for $R32tet_{32}$. The latter protein does have a considerable immunogenicity by itself, but only at higher doses than those used in these experiments. Our results demonstrate that liposomes, lipid A, and ALUM may be considered as additive adjuvants for stimulating an immune response to a very small quantity of an immunogenic protein. Our preliminary results with either small or large doses of antigen indicate that over a long period of time the combined effects of liposomes, lipid A, and ALUM continue to produce a higher and more prolonged immune response than that observed with other combinations of components.

The experiments observed in this study with rabbits provide a promising expectation that enhanced efficacy might be achieved by using a vaccine mixture containing liposome-encapsulated antigen. It is likely that useful information in this regard will be derived by immunization of monkeys. In the clinical trial with $R32tet_{32}$, protection was achieved only in the group of high-dose volunteers that had the highest antibody titers. Our preliminary data with monkeys (Fig. 6) suggest the possibility that the combined effects of liposomes, lipid A, and ALUM may induce antibody responses in humans greater than those induced by ALUM alone. Further studies of long-term immunity induced by scaled-up batches of liposomes prepared under good manufacturing practice conditions are now ongoing with monkeys.

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