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1. REPORT NUMBER		2. SESSION NO.		READ INSTRUCTIONS BEFORE COMPLETING FORM	
4. TITLE (and Subtitle)		3. RECIPIENT'S CATALOG NUMBER		5. TYPE OF REPORT & PERIOD COVERED	
AD-A224 014					
Liposomes as Carriers of vaccines: Development of a liposomal malaria vaccine		6. PERFORMING ORG. REPORT NUMBER		7. CONTRACT OR GRANT NUMBER(s)	
7. AUTHOR(s) C.R. Alving, R.L. Richards, M.D. Hayre W.T. Hockmeyer, and R.A. Wirtz		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS		12. REPORT DATE	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Division of Biochemistry Walter Reed Army Institute of Research Washington, DC 20307		11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Comm. Ft. Detrick, Frederick, MD		13. NUMBER OF PAGES	
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) Walter Reed Army Institute of Research Washington, DC 20307		15. SECURITY CLASS. (of this report) UNCLASSIFIED		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE	
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited					
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)					
18. SUPPLEMENTARY NOTES					
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Liposomes, Carriers, Vaccines, Malaria					
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) This paper describes the development of a liposome vaccine against <u>Plasmodium falciparum</u>					

From: IMMUNOLOGICAL ADJUVANTS AND VACCINES
Edited by Gregory Gregoriadis, Anthony C. Allison,
and George Poste
(Plenum Publishing Corporation, 1989)

LIPOSOMES AS CARRIERS OF VACCINES: DEVELOPMENT OF A LIPOSOMAL MALARIA
VACCINE

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FATE OF LIPOSOMES IN VIVO

Liposomes that have been injected parenterally into animals have a well-known natural tendency to be ingested rapidly and in large amounts by macrophages. Uptake of liposomes by macrophages has often been cited as a potential hurdle that could theoretically block applications of liposomes as drug carriers for certain purposes. However, the macrophage itself has served as a target for delivery of liposome-encapsulated drugs and immunomodulators, particularly for treatment of infectious diseases and cancer. (Alving, 1983, 1989; Fidler, 1985; Swenson et al, 1988). It is certainly true that overcoming of the macrophage as an "obstacle" can be difficult, but several reports have indicated that increased blood circulation time and distribution of liposomes to certain tissues can be achieved by the use of special biophysically or biochemically tailored liposomes. (Hwang et al, 1980; Gregoriadis et al, 1982; Allen and Chonn, 1987; Papahadjopoulos and Gabizon, 1987; Gabizon and Papahadjopoulos, 1988).

DELIVERY OF LIPOSOMES TO ANTIGEN PRESENTING CELLS

Although delivery of liposomes to cells other than macrophages may pose a considerable challenge, in the field of immunology and particularly in the area of vaccine development capture of liposomes by macrophages is not a detrimental phenomenon. In fact, uptake of liposomes by macrophages is highly advantageous to the immune response and greatly facilitates the use of liposomes as carriers of antigens and vaccines. The macrophage is one of a limited number of cell types that are known as "antigen presenting cells" (APC) or "accessory cells", and accumulation of liposomal antigens and adjuvants within macrophages stimulates the humoral immune response to many antigens (Alving, 1987).

Many of the details about the characteristics and functions of APCs are still controversial, but compelling arguments have led to widespread agreement that the humoral immune response to most antigens requires the intervention of APCs (reviewed by Unanue, 1984, and Unanue and Allen, 1987). The APC is characterized by the presence of class II major histocompatibility antigen molecules (Ia antigen) on its surface, and only three major cell types express Ia antigen: macrophages, B cells, and dendritic cells of

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lymphoid tissue and skin. The immunogenic material is internalized by the APC where it may be partially degraded, although the absolute requirement for such degradation has not been fully resolved. The immunogenic epitopes form a complex with Ia antigen molecules, and the complex is transported to the cell surface. In the next stage of the immune response the antigen-Ia complex (or individual constituents thereof) on the APC surface interact specifically with CD-4-positive T-helper cell lymphocytes. The APC and the T-helper cell both are secretory cells, and among the important resulting immunologic products that influence the humoral immune response are included interleukin-1 (from the APC) and interferon- γ (from T-helper cells).

As mentioned earlier, three major cell types express Ia antigen and can serve as APCs. There has been debate on the relative importance or prominence of each type of APC; for example, different relative emphasis has been placed by different investigators on the role of macrophages (Unanue, 1984; Unanue and Allen, 1987) or dendritic cells (Steinman and Nussenzweig, 1980; Tew et al, 1980). Regardless of the relative importance of macrophages compared to dendritic cells in the immunologic scheme of the body, it seems likely that macrophages serve predominantly, or even exclusively, as APCs for liposomal antigens. Several lines of evidence support this conclusion. First, as noted earlier liposomes are ingested avidly by macrophages, but are taken up only slightly, if at all, by most other cells under normal *in vivo* circumstances. In contrast, dendritic cells do not serve as endocytic cells (Steinman and Nussenzweig, 1980) and it is unlikely therefore that dendritic cells would capture liposomes to any significant extent. Second, depletion of macrophages by treatment of animals with carageenan suppressed the ability of the animals to produce an immune response to a liposomal antigen (Shek and Lukovich, 1982). Third, in an interesting experiment liposomes containing a poorly immunogenic antigen (bovine serum albumin) were first ingested by macrophages *in vitro*. The macrophages containing the liposome-encapsulated albumin were then injected into mice and a murine humoral immune response to the liposomal albumin was observed (Beatty et al, 1984).

IMMUNE RESPONSE TO LIPOSOMAL ANTIGENS

Liposomes have been used to induce humoral immunity to numerous liposomal protein antigens (reviewed by Alving, 1987). There has been considerable investigation of the biophysical parameters that influence the immune response. For example, it is likely that liposome-encapsulated and surface-bound antigens are both effective for inducing antibodies. It is also possible that very small liposomes are somewhat more effective for inducing antibodies than large ones, but obtaining very small liposomes can sometimes pose inconvenient technical problems in formulating vaccines because care must be taken to avoid altering the chemical structure of the antigen. With individual antigens differences in the potency of the immune response have also been noted with liposomes containing different net surface charges.

In most but not all cases the antibody response induced by liposomes is enhanced if the liposomes contain an adjuvant (Alving, 1987). Immunity is also increased by mixing or emulsifying the liposomes with certain nonliposomal adjuvants. In our own experience either Freund's complete adjuvant or aluminum hydroxide (alum) can enhance immunity. In the case of alum, additive or synergistic immunostimulating effects were observed when nonliposomal alum was used in combination with liposomal lipid A to stimulate humoral immunity to a liposomal malaria antigen (Richards et al, 1988).

In summary, a considerable amount of information has now been developed on APCs, and it is well-known that liposomes are avidly delivered to at least one APC, the macrophage. The physical, immunologic, and metabolic

interactions of liposomes with macrophages have also been carefully studied. It is therefore logical that liposomes should be proposed as carriers of antigens for vaccines. In the past, limited supplies of useful antigens posed barriers to development of practical vaccines. However, we are currently examining synthetic antigens derived from malaria parasites as potential candidates for a liposomal malaria vaccine.

MALARIA SPOROZOITE VACCINE

In the life cycle of the malaria parasite, the malaria organism is injected into the bloodstream of the mammalian host by the bite of the female anopheline mosquito. The form of the organism that is injected by the mosquito is known as the sporozoite. After entry into the blood the sporozoite rapidly disappears from the blood due to uptake by the liver. Over a period of days it then develops into a different form before reappearance in the blood.

During the past few years, several laboratories have undertaken programs to manufacture and test potential synthetic vaccines to induce humoral immunity against the sporozoite stage of the human malaria parasite Plasmodium falciparum. The feasibility of developing an effective vaccine against the sporozoite form of P. falciparum has been demonstrated in several ways. A successful clinical trial utilizing irradiated sporozoites as antigens demonstrated that protective immunity could be achieved in humans (Clyde et al, 1975). Furthermore, protection against sporozoite-induced infection in animals or humans was correlated with the presence of a high titer of polyclonal antibodies to sporozoites (Clyde et al, 1975; Nussenzweig et al, 1969; Rieckmann et al, 1979) and protection was even obtained by passive infusion of monoclonal antibodies in animals (Potocnjak et al, 1980). The major antigen responsible for inducing protective immunity is a protein that covers the surface of the sporozoite, the circumsporozoite (CS) protein. A positive correlation was observed between anti-CS protein antibodies among individuals living in endemic areas and protection against sporozoite challenge (Hoffman et al, 1986).

The gene encoding the CS protein of P. falciparum has been cloned (Dame et al, 1984). A region in the middle of the protein which contains repeating tetrapeptides is thought to be capable of inducing protective immunity (Dame et al, 1984; Young et al, 1985; Ballou et al, 1985; Zavala et al, 1985). A positive correlation was observed between antibody titers and protection against sporozoite challenge after immunizing humans with a vaccine containing a recombinant protein (R32tet₃₂) derived from the structure of the repetitive region of the CS protein (Ballou et al, 1987).

The available evidence therefore suggests that protective immunity could theoretically be achieved by inducing high titers of antibodies to the CS protein. However, considerable complexity has been observed in the immune response to the CS protein. Titers of naturally-occurring antibodies to CS protein in endemic areas do not correlate well with protection against new malaria infections that occur after treatment for malaria (Hoffman et al, 1987). Lack of protection may be related to immunosuppression that apparently is induced against the repeating tetrapeptide region of the CS protein by intracellular parasites (Webster et al, 1987). When mice were immunized with either irradiated sporozoites or with recombinant or peptide vaccines against the repeat region of the CS protein from P. berghei, protection against experimental infection with malaria was greater, irrespective of antibody titer, after immunization with the irradiated sporozoites (Egan et al, 1987). The latter study suggests that cell-mediated immunity could contribute to an immune response against sporozoites.

Although the antibody titer against the sporozoite or CS protein is not necessarily predictive of protection in the course of natural infections, the feasibility of obtaining a useful vaccine by artificially inducing a strong humoral immune response has not yet been tested successfully in humans. Vaccine trials with a recombinant antigen ("falciparum sporozoite vaccine number 1", or FSV-1) or with a peptide antigen containing epitopes derived from the repeat region of the CS protein did not induce high titers of antibodies in human volunteers (Ballou et al, 1987; Herrington et al, 1987). The numbers of sporozoites experimentally used to test the efficacies of the vaccines in the immunized volunteers were quite high, but despite high sporozoite doses partial or complete protection was still observed among individuals having the highest antibody titers (Ballou et al, 1987; Herrington et al, 1987). The current research challenge, therefore, is to devise methods that can induce consistently high titers of anti-CS protein antibodies by using synthetic vaccines containing repetitive peptides from the CS protein. Recent evidence has suggested that potential vaccines containing liposomes with recombinant or peptide sporozoite antigens can induce titers of antibodies in rabbits and monkeys to CS protein that are substantially higher than those obtained with current vaccines (Alving et al, 1986; Richards et al, 1988).

DEVELOPMENT OF A LIPOSOMAL SPOROZOITE VACCINE

The initial studies in our program were performed with liposomes containing an antigen consisting of bovine serum albumin (BSA) conjugated to 16 mer peptides derived from the structure of the repetitive region of the CS protein. The 16 mer peptides contained 4 asparagine-alanine-asparagine-proline tetrapeptides. Murine antibodies against this antigen were obtained by utilizing Freund's complete adjuvant during the immunization procedure. The antibodies that were produced by this method blocked invasion of human hepatoma cells in vitro (Ballou et al, 1985).

Upon injection of the peptide-BSA conjugate by itself into rabbits (without adjuvant or liposomes), little or no immunogenic activity was detected as measured by ELISA (Fig. 1). In contrast, liposomes containing the peptide-BSA conjugate induced a potent humoral immune response, and the activity was increased further when the liposomes contained lipid A (Fig. 1).

When the sporozoite is injected by the bite of a mosquito into the blood of a host animal it enters the liver within a very short period, usually within a few minutes. It is currently presumed that it is only during this brief period when the sporozoite resides in the blood that the antibodies can gain access to the sporozoite and prevent continuation of the malaria infection. Therefore a major goal of a sporozoite immunization scheme is to maintain a high titer of antibodies over a long period of time, preferably for at least 6 to 12 months. As shown in Fig. 2, after immunization with liposomes containing peptide-BSA and lipid A at 0, 4 and 28 weeks, activity was still present at maximal levels more than 55 weeks after the primary immunization.

Further work in this program has utilized the same recombinant protein antigen (R32tet₃₂) that was employed in the original FSV-1 clinical trial (Ballou et al, 1987). The R32tet₃₂ antigen contains 32 tetrapeptide repeats from the CS protein, and also contains a tail consisting of 32 amino acids derived from the tetracycline resistance gene of the cloning vector (Young et al, 1985; Wirtz et al, 1987). In a series of experiments with R32tet₃₂ involving both rabbits and rhesus monkeys we have now reached the conclusion that among the combinations that we have tested, the most potent immunogenic formulation consists of an alum-liposome mixture in which the liposomes

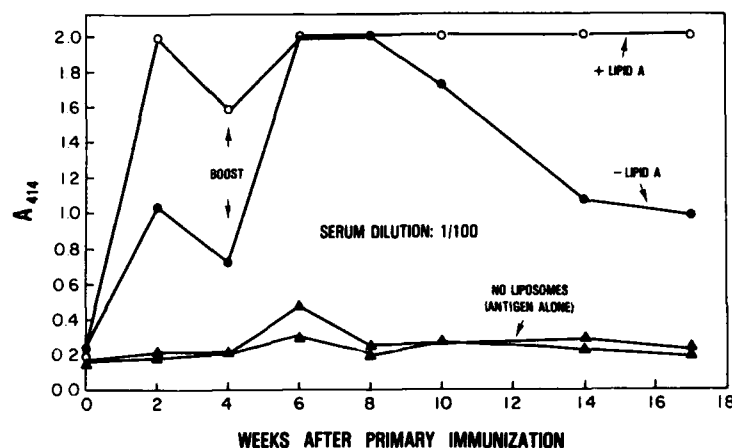


Fig. 1. Immune response against malaria sporozoite peptide-BSA conjugate in rabbits. Antibody activity was detected by enzyme-linked immunosorbent assay (ELISA) with absorbance at 414 nm using a 1/100 dilution of serum. The maximum measurable response (A_{414}) was 2.0. (See Alving et al, 1986 for details of immunization and assay.)

contain both the R32tet₃₂ and lipid A [abbreviated as L(Ag + LA) + alum] (Richards et al, 1988). An example of results that were typical both for rabbits and monkeys is shown in Fig. 3. The highest response occurred with L(Ag + LA) + alum and the lowest response occurred with Ag (R32tet₃₂) alone. The vaccine containing only alum (Ag + alum) gave immunogenic effects at an intermediate level. In monkey experiments, direct comparison of the liposomal formulation with the actual vaccine that was used in the FSV-1 human trial confirmed the same relative ranking of activities. Depending on the dose of vaccine used, and on the date of observation, in monkeys the optimal liposomal vaccine was 15 times to 500 times more potent as an immunogenic formulation than the vaccine used in the FSV-1 human trial.

In summary, it appears from the above results that the liposomes have an ability to serve as an effective carrier for synthetic antigens that are derived from the structure of the CS protein on the surface of the *P. falciparum* sporozoite. The liposomes also have an inherent adjuvant activity; that is, they induce a higher immune response to synthetic peptides or proteins from sporozoites than would otherwise be observed by other methods of immunization. It is reasonable to presume that the adjuvant activity of liposomes may be due to a combination of factors. These factors may include a focussed and enhanced delivery of the antigen to an APC (macrophage) and protection of the antigen from metabolic destruction at other sites in the body that do not participate in the immune response.

There is another possible mechanism by which the liposome might serve as an adjuvant, namely by overcoming immunosuppression that might otherwise be induced by injection of the antigen. Careful inspection of Figs. 2 and 3 reveals that in each case the animals injected with antigen alone (without liposomes) only expressed antibody activity starting between 35 and 40 weeks after initial immunization. It is unlikely that this antibody response was due to a recent immunization dose since the previous immunization dose was either at 28 weeks (Fig. 2) or 4 weeks (Fig. 3) after initial immunization. A similar pattern has also been observed in a preliminary experiment with monkeys. It is possible that initial immunization with either the peptide (Fig. 2) or the recombinant protein (Fig. 3) induces a normal immune response but also simultaneously induces proliferation of suppressor lympho-

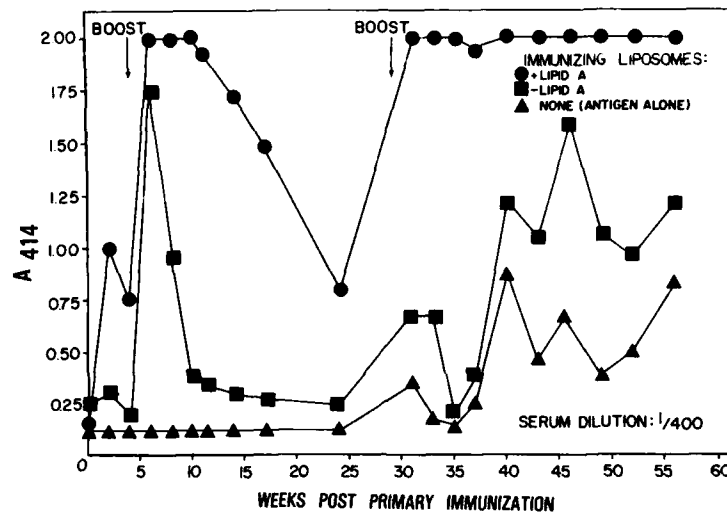


Fig. 2. Long term antibody activity after immunizing rabbits with peptide-BSA conjugate. The rabbits were the same as those in Fig. 1, but a 1/400 dilution of serum was used instead of 1/1000.

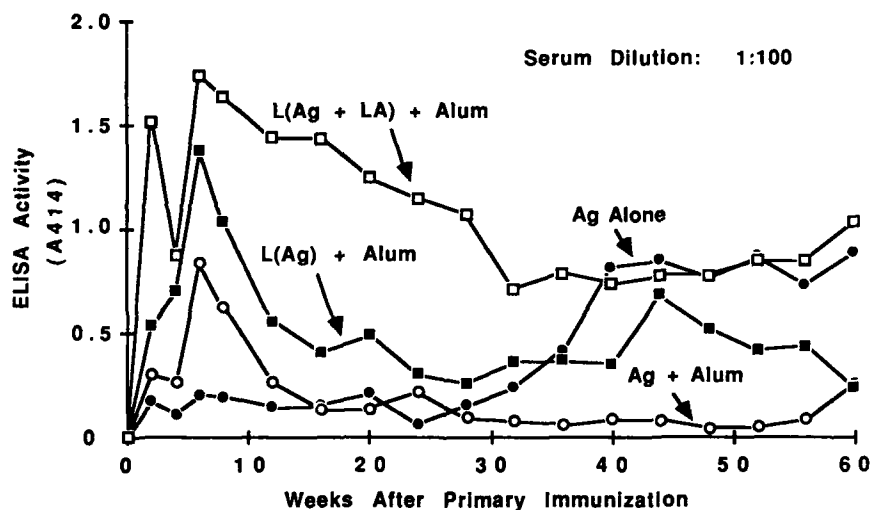


Fig. 3. Effects of alum, lipid A, and liposomes on mean antibody activities of rabbits injected at 0 and 4 weeks. Each point represents the mean ELISA antibody response after subtraction of the preimmune value of a serum dilution of 1:100 for six (Ag + Alum) or four (all other groups) rabbits. Ag alone, free R32tet₃₂; Ag + Alum, 32tet₃₂ absorbed with alum; L(Ag) + alum, alum absorbate of R32tet₃₂ encapsulated in liposomes lacking lipid A; L(Ag + LA) + alum, alum absorbate of R32tet₃₂ encapsulated in liposomes containing lipid A. (From Richards et al, 1988.)

cytes. The spontaneous disappearance of the suppressor effect 35 to 40 weeks later may explain the unexpected expression of antibody activity that was observed at that time.

The unique way in which liposomal antigens are handled, by rapid entry into a phagocytic APC, may cause a bypass of the normal mechanism that the body uses for induction of suppressor lymphocytes. Although this suppressor cell concept is still speculative, it is compatible with immunosuppression against R32tet₃₂ that is seen in the course of natural infections with malaria in humans (Webster et al, 1987), and it could help to explain the great difficulty that has been observed in producing high titers of antibodies to synthetic sporozoite antigens in humans.

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