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Immunization of experimental monkeys against *Plasmodium falciparum*: use of synthetic adjuvants*

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*The replacement of Freund's adjuvant by a possible safe adjuvant for effective immunization of owl monkeys (*Aotus trivirgatus griseimembra*) against a human malaria parasite,* Plasmodium falciparum, *has been investigated. Experiments involved the use of two synthetic adjuvants: MDP (*N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamine) and stearoyl-MDP (6-O-stearoyl-*N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamine). In both cases,* P. falciparum *merozoites obtained through short-term* in vitro *cultivation were used as antigen. MPD was used as adjuvant in 5 owl monkeys; 2 control monkeys died and of the 3 experimental monkeys only 1 survived. In contrast, in another experiment where stearoyl-MDP was used as adjuvant, there was 100% protection of 4 immunized monkeys against a challenge with the homologous strain of* P. falciparum. *The results of the second experiment are encouraging for the development of an effective and safe vaccine for human malaria.*

Attempts to find a vaccine against malaria began half a century ago but gave way to searches for new drugs during the Second World War and to anti-mosquito programmes during the post-war years. However, resistance to drugs and insecticides and lack of money have reduced the prospects of malaria eradication and thoughts have again turned to the possibility of developing a malaria vaccine (*1*).

Four different kinds of vaccines are currently under investigation: exoerythrocytic merozoites raised in tissue culture (*2*), irradiated sporozoites from the mosquito (*3–5*), extracts from blood schizonts (*6–9*), and emulsified erythrocytic merozoites (*10, 11*). Most of the above studies have been made on bird, rodent, and monkey malarias. This report deals with studies made on a human malaria parasite, *Plasmodium falciparum*.

A year ago a report was published from this laboratory (*12*) showing the first successful immunization of *Aotus trivirgatus griseimembra* monkeys against *P. falciparum* infection. This result was rapidly confirmed (*13*). In these studies the use of Freund's complete adjuvant was found to be essential for effective immunization. The use of Freund's adjuvant in man is not considered safe because serious side-effects have been widely reported: potentiation of plasma cell tumours in mice, induction of autoimmune reactions, formation of disseminated focal granulomata, and long-term persistence of mineral oil in animals (*14–18*). The development of an immunologically satisfactory and pharmacologically acceptable adjuvant is of prime importance to the development of an acceptable human malaria vaccine. The synthesis of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP) has been achieved recently in two laboratories (*19, 20*) and it has been shown to be capable of replacing the whole tubercle bacilli and of enhancing the immune response of an animal against an antigen when injected with Freund's incomplete adjuvant. However, before this compound could be used for immunization in man it would be necessary to eliminate the mineral oil, which is partly responsible for undesirable side-reactions. A recent report from one of our laboratories (*21*) has shown that the replacement of the primary hydroxyl group at the C-6 position of MDP by a lauroyl, stearoyl, or docosanoyl group gave an MDP derivative with adjuvant activities. This report describes the results of immunization experiments in *Aotus trivirgatus griseimembra* with *P. falciparum* antigen using MDP and modified MDP as adjuvants.

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MATERIALS AND METHODS

Preparation of antigen

The Uganda–Palo Alto strain (FUP) of *P. falciparum* used in this study is maintained in the laboratory by serial passages of blood-induced infections in owl monkeys (*22*) and by continuous *in vitro* culture (*23*). The antigen was prepared by short-term *in vitro* cultivation of the FUP strain of *P. falciparum* derived from infected owl monkeys. RPMI 1640 medium supplemented with fetal calf serum (FCS) and fatty acid-free bovine albumin (FAF albumin) was used for short-term *in vitro* cultivation of *P. falciparum*. Parasitized blood from owl monkeys was cultured in sterile 500-ml side-arm flasks fitted with stoppers with entry ports for a gas mixture containing 90% N_2, 8% CO_2, and 2% O_2. Seven millilitres of heparinized, parasitized blood, washed twice with medium, were introduced into each culture flask containing 63 ml of RPMI 1640, 8.8 ml of FCS, and 6 ml of FAF albumin (12.5 g/litre). The medium was changed at approximately 12 and 24 hours of incubation. At the end of 35–40 hours' incubation, most of the parasites had developed to mature segmenters containing fully developed individual merozoites. These mature segmenters were concentrated and harvested relatively free of other cellular elements as described previously (*24*). In the final preparation, as described in an earlier report (*12*), the antigenic material consisted of 50–60% segmenters containing individual merozoites and the remainder consisted of other developmental stages of the parasite. The antigen was stored at −20 °C.

Adjuvants and preparation of vaccine

MDP-peanut (groundnut) oil. MDP was synthesized in one of our laboratories (*20*). 1.5 ml of Arlacel A[a] was added to 8.5 ml of peanut oil. The mixture was autoclaved and stored at 4 °C. Three hours before vaccinating the experimental animals, 0.5 ml of the parasite antigen was mixed thoroughly with 0.5 ml of the peanut oil carrier containing 125 μg of freshly added MDP. Details of the composition of the vaccine and the vaccination schedule are given in Table 1.

Stearoyl-MDP and liposomes. 6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine was synthesized in one of our laboratories (*21*). The adjuvant-incorporated liposomes were prepared following the description of Inoue (*25*) with some modifications: 10 μmol of cholesterol (Grade 99 + %)[a] and 10 μmol of lecithin (dipalmitoyl-DL-phosphatidyl choline, Grade I approximately 99%)[a] were dissolved in 5 ml of chloroform in a 10-ml round-bottomed flask. All the chloroform was removed by means of a rotary vacuum evaporator below 30 °C. The adjuvant (0.025, 0.5, or 1.0 mg) in 0.5 ml of phosphate-buffered saline was added to the lipid-coated flask. Liposomes were then prepared by sonication at 0 °C under argon. The vaccine was prepared by thoroughly mixing the adjuvant-incorporated liposomes with antigen, using a double-hubbed needle and two syringes. Further details of the composition of the vaccine and the vaccination schedule are given in Table 2.

[a] From the Sigma Chemical Co., St Louis, MO, USA.

Table 1. Summary of vaccination studies in owl monkeys against *Plasmodium falciparum* using MDP as an adjuvant

Aotus monkey No	Composition of vaccine	Pre-patent[a] period (days)	Peak parasitaemia (%)	Death or survival
A232	Control (saline)	3	60 6	died on day 14
A231	Control (MDP + peanut oil)[b]	3	48 8	died on day 14
A200	Ag + MDP + peanut oil[c]	7	90 4	died on day 13
A201	Ag + MDP + peanut oil[c]	11	8 4	survived
A222	Ag + MDP + peanut oil[c]	10	44 4	died on day 26

[a] A232, A231, and A222 were challenged with 2 6 x 10^4 FUP parasites while A200 and A201 were given 5 x 10^4 FUP parasites

[b] 125 μg of MDP in 0 5 ml of peanut oil given intramuscularly on days 0, 12, 33, and 69.

[c] FUP-merozoite-enriched antigen emulsified with 125 μg of MDP in 0.5 ml of peanut oil given intramuscularly on days 0, 12, 33, and 69.

RESULTS AND DISCUSSION

Immunization studies using MDP as an adjuvant

Five monkeys (*Aotus trivirgatus griseimembra*) weighing approximately 800 g each were used in this experiment (Table 1). Two monkeys (No. A232 and A231) were used as controls; the other 3 (No. A200, A201, and A222) were immunized with *P. falciparum* (FUP strain) merozoites emulsified with an equal volume of purified peanut oil containing 125 μg of MDP. This vaccine was administered intramuscularly 4 times to each of the experimental monkeys, a total of 2.88 mg of antigen protein being given to each animal. Immunization never produced a detectable infection. On day 111, 6 weeks after the fourth vaccination, all 5 monkeys were challenged intravenously, with 1 x 10^3 parasites (FUP strain of *P. falciparum*). None of the 5 animals developed a patent parasitaemia, as monitored by daily thick films. On day 31, 4 weeks after the first challenge inoculum, all animals were again challenged with 5 x 10^4 FUP parasites. Seven days after the second challenge, No. A200 became patent and died 6 days later with a >90% parasitaemia. Monkey A201 became patent on day

Table 2. Immunization of owl monkeys against *Plasmodium falciparum* (Uganda-Palo Alto (FUP) strain) malaria

Monkey No	Composition of vaccine[a]					Number of infected erythrocytes in the challenge inoculum (day 45)[f]
	KGS[b] per injection (ml)	Adjuvant[c] plus liposome[d] (ml)	Parasite protein (mg) in 0.5 ml of KGS (injection 1, day 0)[e]	Parasite protein (mg) in 0.5 ml of KGS (injection 2, day 28)[e]	Total parasite protein (mg)	
A 303	–	–	–	–	–	7.5×10^5
A 291	1.5	–	–	–	–	7.5×10^5
A 287	0.5	1.0	–	–	–	7.5×10^5
A 286	–	1.0	1.0	1.86	2.86	7.5×10^5
A 284	–	1.0	1.0	1.86	2.86	7.5×10^5
A 283	–	1.0	1.0	1.86	2.86	7.5×10^5
A 294	–	1.0	1.4	1.4	2.8	7.5×10^5

[a] Adjuvant with liposome plus antigen mixed, using a double-hubbed needle and two syringes, administered intramuscularly in alternate thighs

[b] Krebs glucose saline

[c] Synthetic 6-*O*-stearoyl-*N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamine. A total of 1.5 mg of this adjuvant was given to each of the monkeys A 287, A 286, A 284, and A 283, while only 0.5 mg was given to A 294

[d] Liposome contained lecithin and cholesterol and was prepared following the method of Inoue (*25*)

[e] More than 60% segmenters containing individual merozoites. The remainder of the parasite material consisted of other developmental stages of the parasite

[f] Parasitized blood was obtained from an infected (FUP) owl monkey. The inoculum was given intravenously.

11, developed a peak parasitaemia of 8.4% on day 23, and cleared the infection by day 29, after the second challenge. All other monkeys remained negative. On day 90, 3 months after the first challenge, all surviving monkeys were challenged with 2×10^6 FUP parasites. Both control monkeys were patent on day 3 and died on day 14—No. A232 with a 60.6% parasitaemia and No. A231 with a 48.8% parasitaemia. Monkey A222 became patent on day 10 and succumbed to the infection on day 26 with a 20% parasitaemia. No. A201 developed a very low-grade infection, with less than 0.1% parasitaemia for 9 days, becoming patent on day 4 and clearing the infection by day 12, after the third challenge.

The results of this experiment are similar to, but somewhat better than, those obtained by Voller & Richards (*26*), who failed to protect owl monkeys with Freund's complete adjuvant and formalin-treated infected cells. In our present experiments, monkeys immunized with *P. falciparum* merozoite antigen mixed with MDP–peanut oil showed a delayed onset of parasitaemia, with one monkey (A201) surviving. Attempts to establish the minimum number of parasites needed to infect an owl monkey resulted in a number of unsuccessful challenges. However, these challenges did not alter the course of infection in the controls and should not detract from the survival of monkey A201.

Immunization studies using stearoyl-MDP and liposomes

Seven owl monkeys weighing approximately 900 g each were used in this experiment. The physical characteristics and coloration of these monkeys indicated that they were of phenotype group B, according to the description of Ma et al. (*27*). Using the standard chromosome preparation method of Moorehead et al. (*28*), these monkeys were found to belong to karyotype II as defined by Ma et al. (*27*). The composition of the vaccine and the immunization schedule are summarized in Table 2. Three monkeys (No. A287, A291, and A303) were used as controls; the other 4 monkeys (No. A283, A284, A286, and A294) were immunized with *P. falciparum* (FUP strain) merozoite-enriched antigen mixed with the adjuvant-incorporated liposomes. This material was administered intramuscularly on two occasions, 4 weeks apart, to each of the 4 experimental monkeys. A total of 2.86 mg of parasite protein (1.0 mg on day 0 and 1.86 mg on day 28) was administered to 3 monkeys (A286, A284, and A283). Monkey No. 294 received a total of 2.80 mg of parasite protein (1.4 mg on day 0 and on day 28). Immunization never produced a detectable infection. On day 45, that is, 17 days after the second inoculation, all 7 monkeys were challenged by intravenous injection of 7.5×10^5 parasites (FUP strain of *P. falciparum*) derived from an ongoing infection in an owl monkey.

Thick or thin blood films were made daily to follow the course of infection in all monkeys and the results are summarized in Fig. 1. The two control monkeys (A303 and A287) died within 1–2 weeks after the challenge. Monkey No. 303 died on day 8 with a 60.0% infection and No. A287 died on day 15 with 54.4% in-

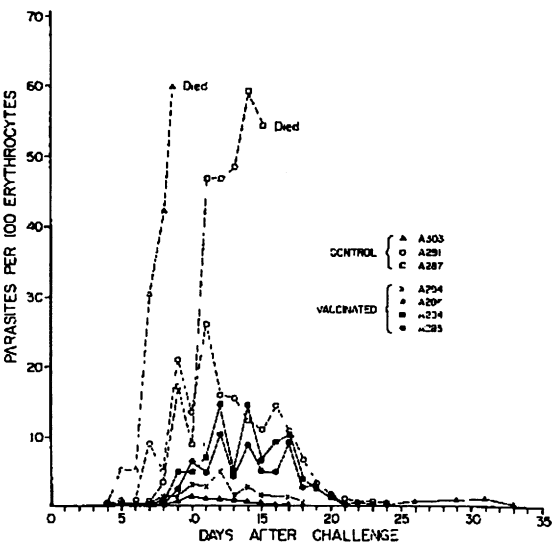

Fig. 1. Course of infection of *Plasmodium falciparum* (FUP strain) in control and vaccinated monkeys (*Aotus trivirgatus griseimembra*).

fection. Although A291 eventually survived the infection, it attained the peak parasitaemia of more than 25%. In contrast, all 4 immunized monkeys survived. In immunized monkeys A286 and A294, low-grade infections lasted for 1 week, while in A284 and A283 the parasitaemia ranged between 5.0 and 14.0% for 1 week. By day 24, A284, A283, and A294 had become negative and no parasites could be detected in A286 by day 32. Up to 6 months after the challenge, all these monkeys remained negative. Although the number of monkeys used in this experiment was small, the difference between the course of infection in immunized and nonimmunized monkeys is indeed very significant. Spontaneous recovery of a control monkey (A291) is a very rare occurrence. Within the last 7 months, blood-induced infections (FUP strain of *P. falciparum*) were initiated in the same manner in 3 other *Aotus trivirgatus griseimembra* (A306, A290, and A275) and all the 3 monkeys showed a typical course of infection and died (Fig. 2). These 3 monkeys were also of karyotype II and phenotype group B.

It is concluded from these results that *P. falciparum* merozoite immunization, using 6-*O*-stearoyl-*N*-acetylmuramyl-L-analyl-D-isoglutamine with liposomes as an adjuvant, protects against homologous infection with intraerythrocytic stages of the normally lethal *P. falciparum* parasites. The administration of *P. falciparum* antigen with adjuvant and liposomes did not produce any reaction at the site of injection. The only side-effect associated with the adjuvant was anorexia for a few days immediately after vaccination, which resulted in some loss of weight. However, all immunized and adjuvant control monkeys regained weight within 2 weeks following immunization. The loss of weight was considerably less in A294, which received only one-third the concentration of the adjuvant given to the other monkeys. This is an important observation, indicating some correlation between the concentration of adjuvant used and the degree of side-effect observed. Experiments are in progress to define the optimal concentration of this new adjuvant that is efficacious and has minimal side-effects.

There are 6 reports to date on studies in owl monkeys of immunization against *P. falciparum*. In two studies, the use of *P. falciparum* ring stages as antigen failed to afford complete protection of the immunized owl monkeys (*26*, *28*). In contrast, in two other studies (*12*, *13*) in which *P. falciparum* merozoites emulsified with Freund's complete adjuvant were used as vaccine, complete protection of the immunized monkeys was obtained. The replacement of Freund's complete adjuvant by MDP did not give 100% protection in two other studies (*23*; W. Trager et al., personal communication). However, the experiments reported above show the first successful replacement of Freund's complete adjuvant by 6-*O*-stearoyl-*N*-acetylmuramyl-L-analyl-D-isoglutamine for effective immunization of owl monkeys (*Aotus trivirgatus griseimembra*) against infection with a human malaria parasite, *P. falciparum*. These results are encouraging and provide good grounds for believing that an effective and safe vaccine for human malaria may be developed in the not too distant future.

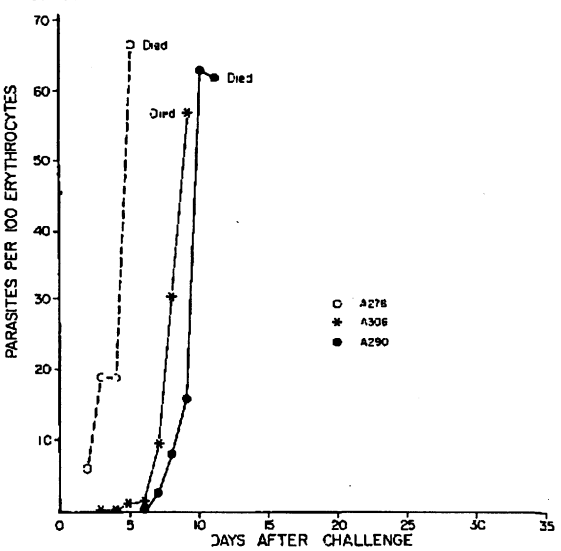

Fig. 2. Course of infection of *Plasmodium falciparum* (FUP strain) in normal *Aotus trivirgatus griseimembra* monkeys.

RÉSUMÉ

IMMUNISATION DE SINGES D'EXPÉRIENCE CONTRE *PLASMODIUM FALCIPARUM*: EMPLOI D'ADJUVANTS SYNTHÉTIQUES

Pour étudier la possibilité de substituer à l'adjuvant de Freund un adjuvant présentant moins de risques d'effets secondaires, on a évalué l'efficacité de la vaccination de singes nocturnes (*Aotus trivirgatus griseimembra*) contre un parasite infectant l'homme, *Plasmodium falciparum*. Les expériences ont été faites avec deux adjuvants synthétiques différents: MDP (*N*-acétyl-muramyl-L-alanyl-D-isoglutamine) et stéaroyl-MDP (6-*O*-stéaroyl-*N*-acétyl-muramyl-L-alanyl-D-isoglutamine). On a utilisé dans les deux cas comme antigène les mérozoïtes de *P. falciparum* obtenus au moyen d'une culture *in vitro* de courte durée. L'expérience avec MDP a porté sur 5 singes nocturnes: les 2 singes servant de contrôles sont morts et un seul des 3 singes soumis à l'épreuve a survécu aux inoculations ultérieures de parasite. Par contre, dans l'expérience utilisant stéaroyl-MDP comme adjuvant, une protection à 100% a été constatée chez les 4 singes vaccinés lorsque ceux-ci ont été exposés à une souche de *P. falciparum* homologue. Quant aux 3 singes servant de contrôles, ils ont contracté la maladie et 2 d'entre eux y ont succombé. Les résultats de la seconde expérience permettent d'envisager avec optimisme les perspectives de mise au point d'un vaccin contre le paludisme humain qui soit à la fois efficace et satisfaisant sur le plan de la sécurité.

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