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Vaccination of Rhesus monkeys (*Macaca mulatta*) against *Plasmodium knowlesi* by the use of nonviable antigen

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This study was part of a long-term project with the ultimate goal of developing a malaria vaccine for use in man. Rhesus monkeys that had been vaccinated with a nonviable antigen given in combination with Freund's adjuvant were protected against a normally lethal challenge of Plasmodium knowlesi. *Use of the antigen alone or in combination with other adjuvants was not successful. The fact that monkeys were protected against an infection that is normally lethal suggests that a similarly prepared antigen might be of use against malaria in human beings.*

Successful vaccination of monkeys against *P. knowlesi* was reported by Freund et al. (1945), Targett & Fulton (1965), and Brown et al. (1970). In each of those experiments either killed infected cells or killed whole parasites were the vaccinating material. The feasibility of vaccinating Rhesus monkeys against *P. knowlesi* with a partially purified plasmodial antigen fraction was briefly reported by D'Antonio et al. (1971). Our study, which confirms and expands those results, has shown that it is possible to immunize animals with only 2 injections of plasmodial antigen in Freund's adjuvant. However, it was not possible to immunize monkeys successfully with antigen alone or in combination with BCG or the synthetic polynucleotide Poly A : U—the two other experimental adjuvants tested. Upon examination of blood smears after inoculation with viable parasites, the animals that were protected demonstrated only slight transient parasitaemia or no parasites at all.

MATERIALS AND METHODS

Animals

Rhesus monkeys were used in all the experiments. The animals were either juveniles (3–4 kg) or adults (6–7 kg), as specified in each experiment. Adults and juveniles were not mixed in any experiment. All monkeys were quarantined and conditioned for at least 2 months before use and were in good health, as determined by the staff veterinarian, at the beginning of each experiment.

Antigen

Antigen was prepared following a modified procedure of D'Antonio (1972) (Table 1). Disintegration of both cells and parasites was carried out in a French Pressure Cell and Press.[4] Parasites were freed from red blood cells at 5.86×10^6 N/m^2 and, after the prescribed washes, the parasites themselves were disintegrated at 1.24×10^8 N/m^2. The supernatant, which was obtained after centrifugation of this material, was subjected to molecular sieve chromatography through Sephadex G-200. The antigen used for vaccination was that recovered from the void volume; 1.0 ml of antigen, alone or combined with adjuvant as indicated, was utilized for each injection.

Adjuvants

Three adjuvants were utilized in an attempt to enhance the effect of the antigen. In Experiment 1, Freund's complete adjuvant[5] was combined with an

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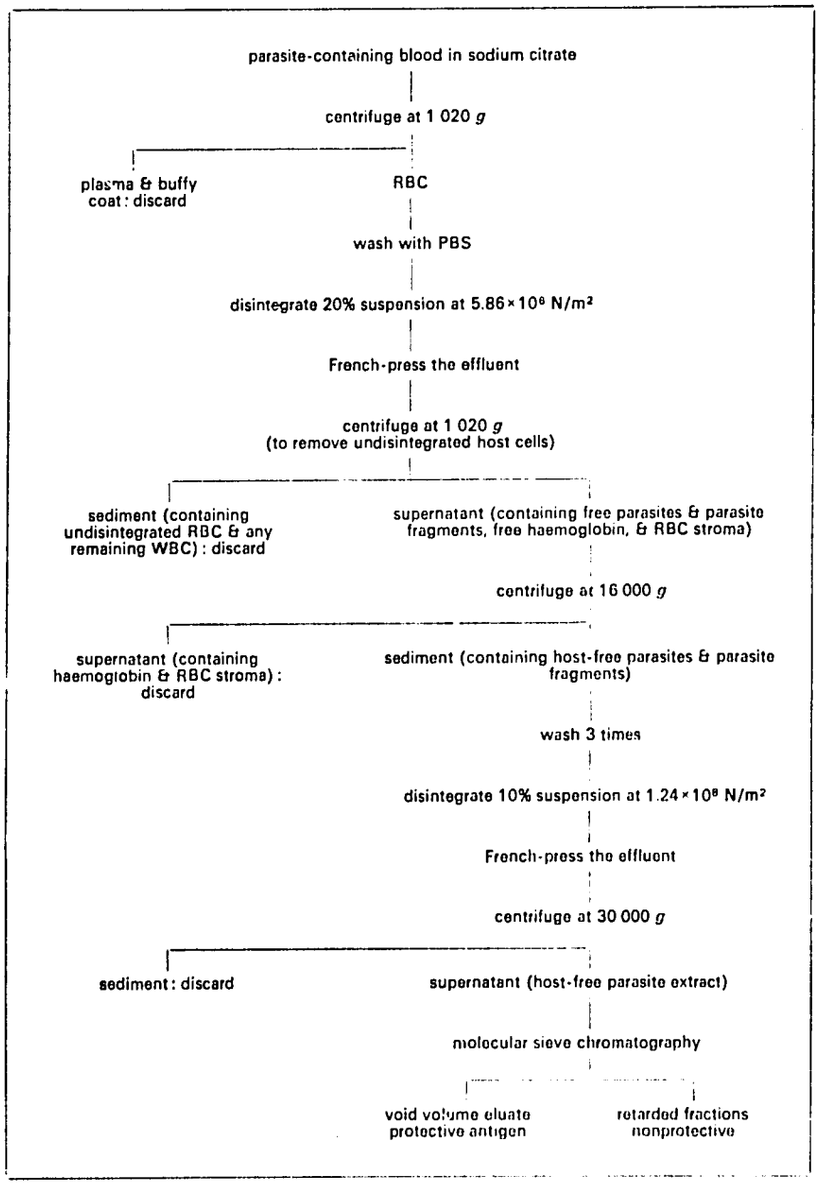
[4] American Instrument Co., Inc., Silver Spring, Md., USA. The pressures at which the instrument was used are indicated in newtons per square metre, in accordance with the ISO international standard.
[5] Difco Laboratories, Detroit, Mich., USA.

Table 1. Flow diagram illustrating the procedure for preparing nonviable *P. knowlesi* antigen with the French Pressure Cell (FPC) technique



equal volume of antigen for the first injection. The second injection consisted of Freund's incomplete adjuvant combined with an equal volume of antigen.

In Experiment 2, a combination of nucleotides and aluminium hydroxide (Poly A : U–Alsol) was used as an adjuvant.[1] For the first injection, 3.0 mg of Poly A were combined with 3.0 mg of Poly U, dissolved in 2.5 ml of pyrogen-free saline (8.5 g/l),

[1] See Acknowledgements.

and stored overnight at 4°C. The mixture was then warmed in an 80°C water bath for 10 min and cooled to room temperature, after which 3.0 ml of antigen were added. The mixture was stored at room temperature for at least an hour. It was then shaken vigorously while 5.0 ml of Alsol No. 3 (aluminium 3.1 g/l) were added to it. The preparation was kept refrigerated until used. For the second injection, the same protocol was followed, the only change being that each animal received 10 mg of Poly A : U.

In Experiment 3, BCG was used as an adjuvant. For this purpose, 3.0 mg of lyophilized organisms were reconstituted in a small quantity of sterile physiological saline and combined with the antigen to be injected.

Injections

All injections were given intramuscularly in the flank. The second injection was given 1 month after the first. When Freund's adjuvant and BCG were used, the first injection was given into the right flank and the second into the left. When Poly A : U was used it was necessary to inject the preparation into both flanks at each injection because of the larger volume of adjuvant.

Challenge

The challenge inoculation was with approximately 1×10^6 *P. knowlesi*-infected red blood cells given intravenously 1 month after the second vaccination. The parasites used for challenge were either from an active infection or from infected blood that had been stored at −90°C.

The percentage of parasitaemia was calculated from giemsa-stained thin blood smears, made daily. Red blood cell counts were made with a Coulter particle counter.[1]

Seven weeks after the initial challenge all surviving animals in Experiment 1 were rechallenged with approximately 1×10^6 *P. knowlesi*-infected red cells.

RESULTS

Experiment 1

Juvenile monkeys weighing 3–4 kg were used exclusively in this experiment. Of the 5 animals vaccinated with Freund's adjuvant and antigen as described (Nos. 4141, 4252, 4253, 4249, and 4168), 3 survived (Fig. 1). One animal (No. 1223) that had been kept unvaccinated as a control but that was challenged at the same time as the other animals died following a normal course of the infection. One animal (No. 4253) of the experimental group showed only slight parasitaemia and most probably died of a heavy bacterial infection that was observed in blood smears just before death. Of the 3 surviving vaccinated monkeys, 2 did not demonstrate parasites in blood smears, whereas 1 had a peak parasitaemia level of 2.6%. The 2 with no demonstrable parasites maintained a normal red blood cell count, whereas the red blood cell count in the animal with a slight infection dropped to an extremely low level before rising back to a normal level (Fig. 2). One of the experimental animals (No. 4168) gave no evidence of being protected by the vaccination. Upon rechallenge 6 weeks after the initial challenge, the red blood cell count in all surviving monkeys remained normal and parasites were not observed in blood smears.

Experiment 2

All monkeys in this experiment were adult females weighing between 6 and 7 kg. Animals vaccinated with BCG and antigen showed no evidence of being protected against challenge with *P. knowlesi* (Fig. 3 and 4). All 3 animals in this group died with very high parasitaemia. Control animals that received BCG alone also died of a fulminating infection. Animals that received antigen without adjuvant also died without indicating any evidence of protection (Fig. 5 and 6).

Experiment 3

All monkeys in this experiment were adult females weighing between 6 and 7 kg. The 3 that were vaccinated with the Poly A : U–Alsol mixture combined with antigen died following a normal course of infection. Parasitaemia at death ranged from 42% to 75% with a concomitant drop in the red blood cell count (Fig. 7 and 8). Control animals that received the adjuvant without antigen also died following a normal course of infection. As a further control in the experiment, a group of animals was included that had received the same schedule of Freund's adjuvant and antigen as in Experiment 1; 2 out of 3 animals in this group survived the challenge with parasitaemia never exceeding 1% (Fig. 9 and 10). One animal demonstrated parasites in its blood for only 1 day and did not show a marked drop in the red blood cell count. The other surviving monkey

[1] Coulter Electronics, Hialeah, Fla., USA.

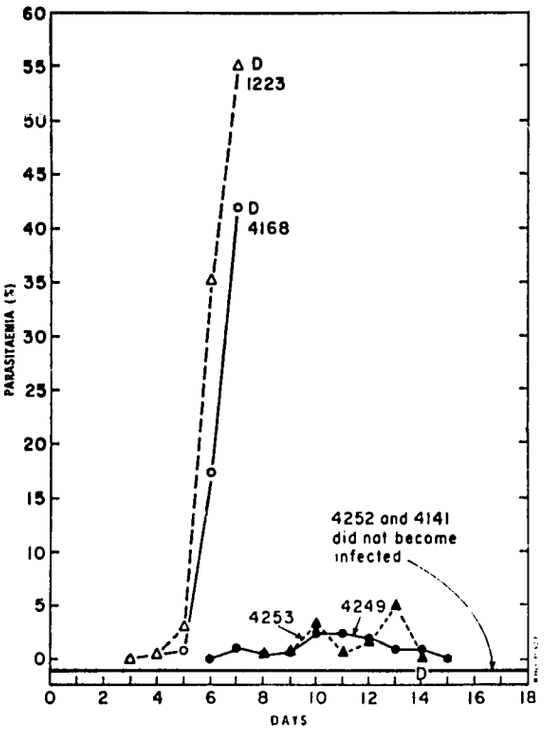


Fig. 1. Parasitaemia in monkeys vaccinated with *P. knowlesi* antigen in combination with Freund's adjuvant and challenged on day 0 with 1×10^6 *P. knowlesi*-infected red blood cells (1 223 : unvaccinated control).

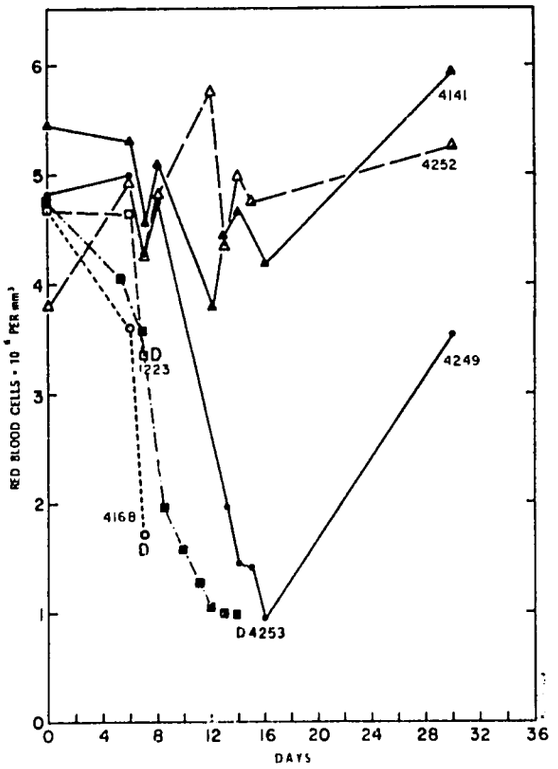


Fig. 2. Red blood cell count of monkeys vaccinated with *P. knowlesi* antigen in combination with Freund's adjuvant and challenged on day 0 with 1×10^6 *P. knowlesi*-infected red blood cells (1 223 : unvaccinated control).

showed a trace infection for 5 days with a noticeable drop in its red blood cell count.

DISCUSSION

The vaccinating material used in this series of experiments was prepared by the method first reported by D'Antonio et al. (1966a), in which the French press was used. At a pressure of $5.68 \times \times 10^{-2}$ N/m², the parasites are released from the red blood cells. As reported by Killby & Silverman (1969), cell disintegration by this technique releases a large proportion of intact parasites that generally lack closely associated red cell membranes. The pressure of 1.24 N/m² utilized in the last step of this procedure disrupts the parasites so that, after final centrifugation to remove whatever unbroken parasites may remain, the material—which after column chromatography will be used for vaccination—is free of whole parasites as assessed by light microscopy. Preliminary studies with the electron microscope demonstrate trilaminate membrane-like structures in this material. Since D'Antonio et al. (1966b) presented serological evidence suggesting that the material is free of significant host cell contamination, there is a likelihood that these membranes are of parasitic origin and actually contain the protective antigen.

This material, as recovered from *P. berghei*-infected mouse blood, has been utilized by D'Antonio et al. (1970) to protect mice against infection with the homologous strain. D'Antonio et al. (1971) subsequently reported that a similar antigen prepared from *P. knowlesi*-infected blood protected monkeys against the homologous strain. In the latter study

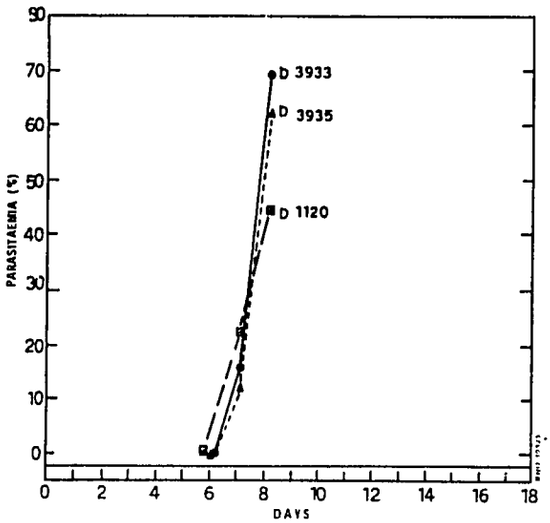


Fig. 3. Parasitaemia in monkeys vaccinated with *P. knowlesi* antigen in combination with BCG and challenged on day 0 with 1×10^6 *P. knowlesi*-infected red blood cells.

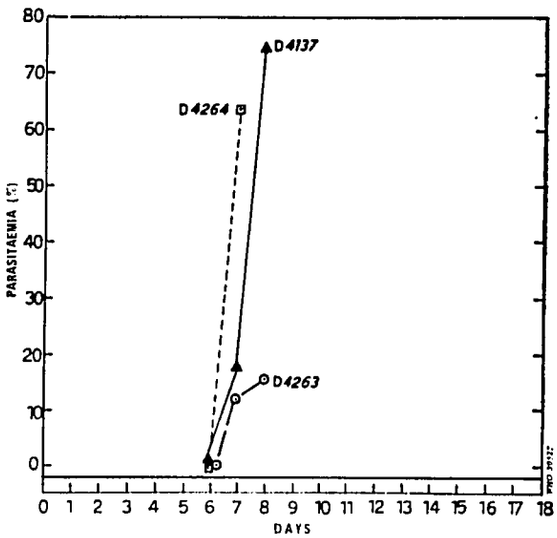


Fig. 5. Parasitaemia in monkeys vaccinated with *P. knowlesi* antigen without adjuvant and challenged on day 0 with 1×10^6 *P. knowlesi*-infected red blood cells.

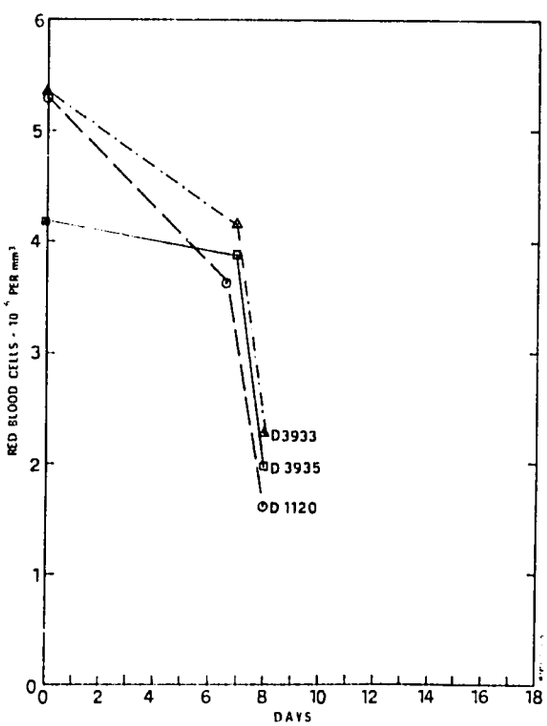


Fig. 4. Red blood cell count of monkeys vaccinated with *P. knowlesi* antigen in combination with BCG and challenged on day 0 with 1×10^6 *P. knowlesi*-infected red blood cells.

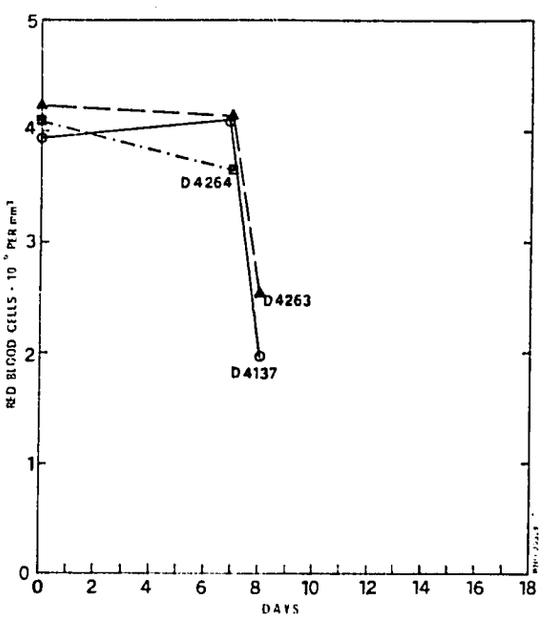


Fig. 6. Red blood cell count of monkeys vaccinated with *P. knowlesi* antigen without adjuvant and challenged on day 0 with 1×10^6 *P. knowlesi*-infected red blood cells.

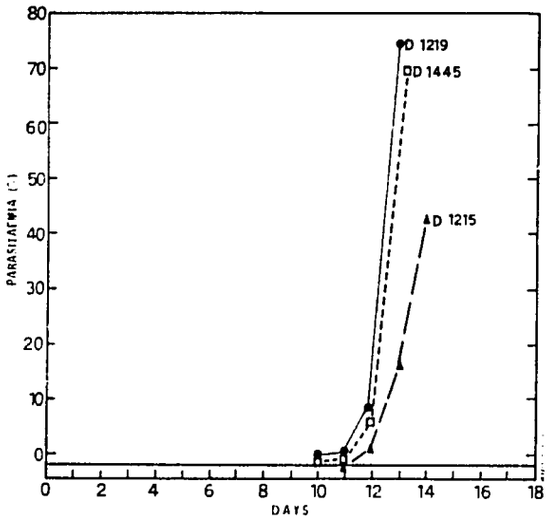


Fig. 7. Parasitaemia in monkeys vaccinated with *P. knowlesi* antigen in combination with Poly A : U-Alsol and challenged on day 0 with 1×10^6 *P. knowlesi*-infected red blood cells.

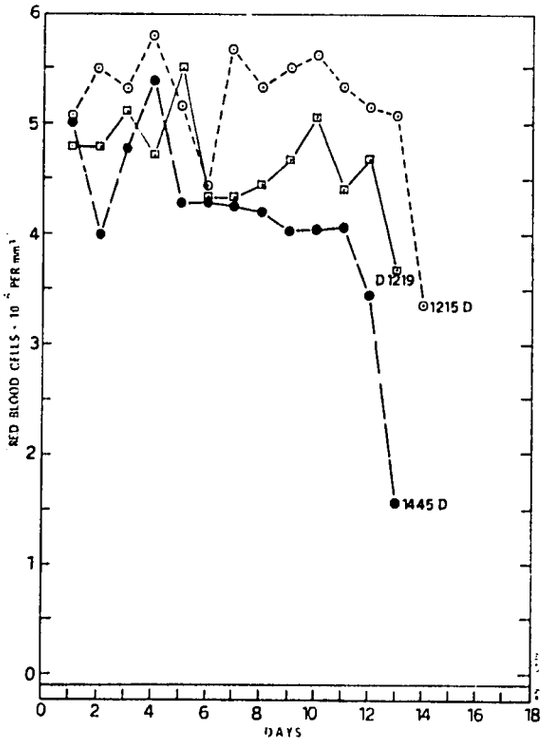


Fig. 8. Red blood cell count of monkeys vaccinated with *P. knowlesi* antigen in combination with Poly A : U-Alsol and challenged on day 0 with 1×10^6 *P. knowlesi*-infected red blood cells.

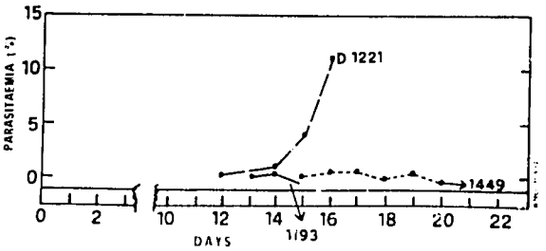


Fig. 9. Parasitaemia in monkeys vaccinated with *P. knowlesi* antigen in combination with Freund's adjuvant and challenged on day 0 with 1×10^6 *P. knowlesi*-infected red blood cells.

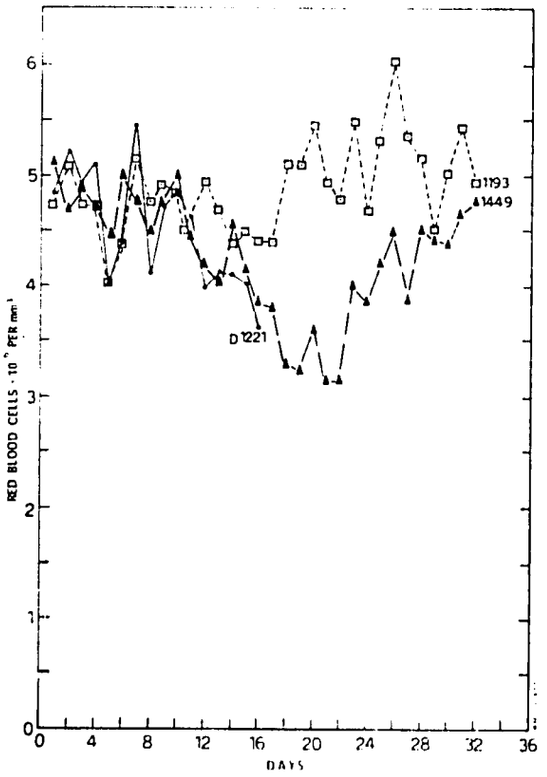


Fig. 10. Red blood cell count of monkeys vaccinated with *P. knowlesi* antigen in combination with Freund's adjuvant and challenged on day 0 with 1×10^6 *P. knowlesi*-infected red blood cells.

only the animals that received antigen intravenously and intramuscularly in combination with Freund's adjuvant were protected. We have now demonstrated that only 2 intramuscular injections of a small quantity of antigen emulsified in Freund's adjuvant are needed to induce good protection in Rhesus monkeys.

So far, owing to the lethal nature of *P. knowlesi* infection in Rhesus monkeys, protection has been measured solely on the basis of survival. Protected monkeys, however, always show a low level of parasitaemia, even when the red blood cell count drops drastically. We have not yet quantified the amount of antigen necessary to induce such protection, although experiments to that end are currently being conducted with lyophilized antigen.

In all experiments carried out by ourselves and other workers, animals injected with antigen alone were not protected (Eaton & Coggeshall, 1939; Targett & Fulton, 1965). As the eventual goal is to produce a vaccine for use in man, an adjuvant was sought which, when combined with antigen, would induce protection but would not produce the serious side effects of Freund's adjuvant. Although BCG and Poly A : U are not commercially used as adjuvants, they have been shown experimentally to be potent stimulators of the immune system. Hiu & Lamiel (1970) reported that a BCG cellular extract was an effective adjuvant, and Morton et al. (1971) and Zbar et al. (1971) reported that immunization with BCG induced a heightened response to tumour cells. We were not able to induce protection by injecting our antigen in combination with BCG. Such inoculation also resulted in the formation of large open abscesses. It is possible that the use of smaller quantities of BCG might reduce the abscess problem. However, as protection was not accomplished with the large quantity used in this study, there seems to be little purpose in pursuing the use of BCG as an adjuvant.

Synthetic polynucleotides can stimulate both cellular and humoral immunity (see review by Braun et al., 1971). However, as small mammals were used as experimental animals in all the reported studies using Poly A : U, the amount of material that must be used in primates has not yet been determined. Braun and his group report that, although initial antibody response to a given antigen is elevated as effectively by Poly A : U as by Freund's complete adjuvant, a prolonged high antibody titre is accomplished more effectively by the latter. This may explain why Poly A : U, when combined with our antigen, failed to induce a protective response.

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RÉSUMÉ

VACCINATION DE SINGES RHÉSUS (*MACACA MULATTA*) CONTRE *PLASMODIUM KNOWLESI* AU MOYEN D'UN ANTIGÈNE NON VIABLE

Au cours d'une série d'expériences, on a utilisé un matériel plasmodique non viable, partiellement purifié, pour immuniser des singes rhésus contre une infection d'épreuve par *Plasmodium knowlesi*. L'antigène, préparé à partir de sang infecté par *P. knowlesi*, ne contenait aucun parasite intact et était exempt de toute contamination par des éléments cellulaires de l'hôte.

Dans un 1er essai, cinq singes ont reçu, à un mois d'intervalle, deux injections intramusculaires de l'antigène additionné d'adjuvant de Freund. Un mois après la dernière injection, ils ont été inoculés par voie intraveineuse par environ 10 000 érythrocytes infectés par *P. knowlesi*. Trois singes ont survécu à cette infection d'épreuve, normalement mortelle: chez deux d'entre eux, aucun parasite n'a été décelé dans le sang périphérique; le 3e a présenté une parasitémie ne dépassant pas 2,6%. Chez l'un des deux singes qui ont succombé, l'infection à *P. knowlesi* a suivi une évolution normale avec une parasitémie de 42% attestant l'échec de la vaccination; chez l'autre, la parasitémie n'a pas dépassé 5% et l'animal est probablement mort d'une infection bactérienne secondaire. Un singe témoin non vacciné est mort après avoir présenté une parasitémie de l'ordre de 55%.

Lors d'autres essais, on a tenté d'immuniser des singes

soit par l'antigène seul soit par l'antigène associé à d'autres adjuvants (BCG ou un mélange de nucléotides et d'hydroxyde d'aluminium). Tous les animaux ayant reçu l'un ou l'autre de ces schémas sont morts, l'infection d'épreuve par *P. knowlesi* suivant son cours normal avec une parasitémie élevée indiquant une absence complète de protection.

Cette étude démontre qu'il est possible de vacciner efficacement des singes rhésus contre *P. knowlesi* à l'aide d'un antigène ne contenant aucun parasite intact. Deux injections de doses relativement faibles de matériel antigénique suffisent. Jusqu'à présent, la vaccination n'est effective que si l'antigène est associé à l'adjuvant de Freund. Des recherches sont en cours pour découvrir un autre adjuvant actif qui pourrait éventuellement être incorporé à une préparation vaccinale destinée à l'homme.

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