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9. ABSTRACT A combination of Adjuvant-65 and Bacillus Calmette-Guerin (B.C.G.) was successfully used as an adjuvant in immunization against <u>Plasmodium knowlesi</u> in Rhesus monkeys (<i>Macaca mulatta</i>). The adjuvant combination was emulsified with a non-viable lyophilized antigen prepared from the blood stages of the parasite. Four monkeys were injected with this mixture on two occasions. Three of the four animals survived a challenge with 2.5×10^5 <u>P. knowlesi</u> -infected red blood cells. All of the control monkeys succumbed to the infection. In the same experiment, four other monkeys were immunized with the same antigen emulsified in Freund's complete adjuvant for the first injection of <u>P. knowlesi</u> , and with the antigen emulsified in Freund's incomplete adjuvant for the second injection. Three of the four animals survived the challenge. In addition to suggesting a new adjuvant for use in malaria vaccination, this is the first report of the successful vaccination against malaria with lyophilized antigen. Its use would be an advantage in tropical areas where storage and shipment of aqueous vaccine is a problem.		
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A NEW ADJUVANT FOR USE IN VACCINATION AGAINST MALARIA

by

Robert H. Schenkel, Edelberto J. Cabrera, Mary L. Barr, and Paul H. Silverman

University of New Mexico, Department of Biology

Albuquerque, New Mexico 87131

A combination of Adjuvant-65 and Bacillus Calmette-Guerin (B.C.G.) has been successfully used as an adjuvant in immunization against Plasmodium knowlesi in rhesus monkeys (Macaca mulatta). The adjuvant combination was emulsified with a non-viable lyophilized antigen prepared from the blood stages of the parasite. Four monkeys were injected with this mixture on two occasions. Three of the four animals survived a challenge with 2.5×10^5 P. knowlesi-infected red blood cells. All the control rhesus succumbed to the infection.

In the same experiment, 4 monkeys were immunized with the same antigen emulsified in Freund's complete adjuvant for the first injection, and in Freund's incomplete adjuvant for the second injection. Three out of the four animals survived the challenge.

Further details and discussion on the possible implications of this study in future malaria vaccinations are included.

A New Adjuvant for Use in Vaccination against Malaria

Previous studies have demonstrated that it is possible to immunize Rhesus monkeys against *Plasmodium knowlesi* with a partially purified nonviable blood stage antigen (Schenkel et al., 1973, Bull World Health Organ 48: 597-604; Simpson et al., 1974, Nature 297: 304-306). Successful vaccinations against *P. knowlesi* using other blood stage antigens have also been reported (Freund et al., 1945, Science 102: 202-204; Targett and Fulton, 1965, Exp Parasitol 17: 180-193; Brown et al., 1970, Exp Parasitol 28: 304-317; Mitchell et al., 1974, Nature 252: 311-313). All of these vaccination systems have required the use of Freund's Complete Adjuvant (FCA). As FCA is not suitable for human use, it was deemed advisable to test other adjuvants which might be suitable and which, when combined with plasmodial antigen, could induce protection. One adjuvant tested, Adjuvant 65 plus Bacillus-Calmette-Guerin (BCG), when emulsified with antigen afforded the same degree of protection as did antigen emulsified with FCA. Adjuvant 65, which has been used in human vaccination systems (Hilleman et al., 1972, Ann Allergy 30: 152-158), substitutes completely metabolizable peanut oil for the mineral oil in Freund's adjuvant. This eliminates the major objection to the use of Freund's in humans which is the apparent long-term persistence of the mineral oil (Freund, 1956, Advan Tuberc Res 7: 130-148).

Lyophilized *P. knowlesi* antigen was prepared and given on the same injection schedule

as previously described (Schenkel et al., 1973, loc. cit.; Simpson et al., 1974, loc. cit.). One milligram of antigen as determined by Lowry's protein analysis was given at each vaccination. Adjuvant 65 was prepared as described by Hilleman et al. (1972, Ann Allergy 30: 177-183). BCG, provided by the University of Illinois BCG Laboratory in a lyophilized state, was reconstituted in sterile distilled H₂O immediately prior to use. Two × 10⁷ plaque-forming units of this material per monkey were added to the Adjuvant 65 immediately prior to emulsification with antigen. One month after the second vaccination all monkeys were challenged intravenously with 2.5 × 10⁵ *P. knowlesi* (II strain) infected red blood cells. Parasitemias were monitored daily by means of Giemsa-stained thin blood films.

Three of four monkeys in the groups vaccinated with FCA and antigen or Adjuvant 65 plus BCG and antigen survived challenge with the normally lethal *P. knowlesi* (Table I). All of the monkeys in the other groups succumbed to the infection. The maximum parasitemias in surviving monkeys were significantly lower than in nonsurvivors. Although no monkeys were injected with BCG alone or with BCG plus antigen, we had not found either of these preparations capable of inducing protection in earlier studies (Schenkel et al., 1973, loc. cit.).

These results, while not ruling out the contributory effect of antibodies in vaccine-induced protection against malaria, suggest that cell-mediated immunity (CMI) is important.

TABLE I. Results after challenge of experimental monkeys with 2.5 × 10⁵ *Plasmodium knowlesi*-infected red blood cells.

Treatment	Number of animals/group	Number of survivors/group	Maximum peak parasitemia of survivors Avg (Range)	Maximum peak parasitemia of nonsurvivors Avg (Range)
Control—No vaccination	4	0	—	39.4 (23.0-63.0)
Freund's adjuvant and <i>P. knowlesi</i> antigen	4	3	7.6 (1.6-12.0)	14.6 (—)
Adjuvant 65 + BCG and <i>P. knowlesi</i> antigen	4	3	3.4 (0.5-6.0)	42.0 (—)
Adjuvant 65 + BCG, no <i>P. knowlesi</i> antigen	4	0	—	38.2 (15.0-66.3)
Adjuvant 65 and <i>P. knowlesi</i> antigen	4	0	—	46.2 (15.0-59.3)
Adjuvant 65, no <i>P. knowlesi</i> antigen	4	0	—	35.1 (18.0-69.0)

Inactivated vaccines normally induce a response that is predominantly humoral in nature (Collins, 1969, *J Bact* 97: 676-683). The antibody response is greatly enhanced when antigen is emulsified in Adjuvant 65 (Woodhour et al., 1964, *Proc Soc Exp Biol Med* 116: 516-522). In this study we did not observe any protective effect when nonviable *P. knowlesi* antigen was emulsified in Adjuvant 65. This indicates that enhanced antibody formation alone is not sufficient to induce protection. Protection was induced, however, by the addition of BCG to the emulsion. In the past several years there have been reports that BCG can nonspecifically, as well as preferentially, stimulate CMI when used as an adjuvant in cell vaccines (Sokal et al., 1972, *Cancer Res* 32: 1584-1589).

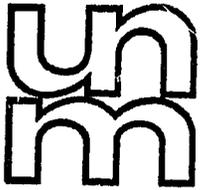
An exception to the rule that inactivated vaccines induce a humoral response is when they are combined with FCA, in which case, CMI, as well as enhanced antibody synthesis, will result (Nelson et al., 1967, *Aust J Exp Biol Med Sci* 45: 113-130). Previous success

in vaccinating against *P. knowlesi* with blood stage antigens has necessitated the use of FCA. This study indicates that an adjuvant which is known to stimulate antibody production cannot induce protection unless combined with BCG, a known stimulator of CMI. Taken together, these results suggest an important role for CMI in vaccine-induced protection against malaria.

In addition to suggesting a new adjuvant for use in malaria vaccination, this is the first report of the successful vaccination against malaria with lyophilized antigen. The use of lyophilized antigen would be an advantage in tropical areas where storage and shipment of aqueous vaccine is a problem.

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Robert H. Schenkel, Edelberto J. Cabrera, Mary L. Barr, and Paul H. Silverman, Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131



THE UNIVERSITY OF NEW MEXICO | ALBUQUERQUE, NEW MEXICO 87131

DEPARTMENT OF BIOLOGY
TELEPHONE 505: 277-3411

September 23, 1975

Mr. Charles J. Dove
Office of Research and
Institutional Grants
Department of State
Agency for International Development
Washington, D. C. 20523

Dear Mr. Dove:

Enclosed are reprints of an article entitled, "A New Adjuvant for Use in Vaccination against Malaria" which appeared in the Journal of Parasitology, Vol. 61, No. 3, June 1975, p. 549-550, by Robert H. Schenkel, Edelberto J. Cabrera, Mary L. Barr and Paul H. Silverman.

An abstract of this paper is also enclosed.

Yours sincerely,

Kay Meyer, Administrative Assistant
Malaria Immunity and Vaccination
Program

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