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VACCINATION OF RHESUS MONKEYS AGAINST MALARIA BY USE OF SUCROSE DENSITY GRADIENT FRACTIONS OF PLASMODIUM KNOWLESI ANTIGENS

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9. ABSTRACT
We wish to report the results of immunity induction trials in the Plasmodium knowlesi-Rhesus monkey system. Thirteen juvenile (1.5-2.0 kg) Rhesus monkeys (Macaca mulatta) were vaccinated with various non-viable fractionation products derived from the plasmodial parasite, P.knowlesi. Samples were prepared from parasitised erythrocytes by differential French Pressure Cell disintegration and centrifugation, Bio-Gel column chromatographic separation, and linear sucrose density gradient fractionation. Two injections of the respective antigen preparations, emulsified in Freund's adjuvant, were given at 4-week intervals, followed 4 weeks later by blood-stage challenge with the homologous P. knowlesi strain. The resulting data reflect a significant difference in peak parasitaemias of the vaccinated monkeys as compared to control monkeys. In this study, mortality figures (antigen in Freud's adjuvant versus control groups) become significant to less than 1% only when the results of the preceding vaccination series are included. Summation of the data produces a total series of twenty-five monkeys. Eleven of seventeen vaccinated animals have survived challenge, while none of the eight control animals have survived (P 1%). In summary, we have demonstrated that a significant decrease in parasitaemia and an increase in survival of Rhesus monkeys infected with P.knowlesi can be induced by the administration of non-viable sucrose density gradient fractions, in Freud's adjuvant, of the void volume preparation used successfully in earlier studies.

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Vaccination of Rhesus monkeys against Malaria by use of sucrose density gradient fractions of *Plasmodium knowlesi* antigens

WE wish to report the results of immunity induction trials in the *Plasmodium knowlesi*-Rhesus monkey system. Thirteen juvenile (1.5-2.0 kg) Rhesus monkeys (*Macaca mulatta*) were vaccinated with various non-viable fractionation products derived from the plasmodial parasite, *P. knowlesi*. Samples were prepared from parasitised erythrocytes by differential French Pressure Cell disintegration and centrifugation, Bio-Gel column chromatographic separation, and linear sucrose density gradient fractionation. Two injections of the respective antigen preparations, emulsified in Freund's adjuvant, were given at 4-week intervals, followed 4 weeks later by blood-stage challenge with the homologous *P. knowlesi* strain. The resulting data reflect a significant difference in peak parasitaemias of the vaccinated monkeys as compared to control monkeys.

Outlined in Fig. 1 and its legend are the preparatory procedures used in obtaining the various parasite fractions which were subsequently tested for antigenic potential. Experimental treatments and results are described in Table 1 and Fig. 2.

The first injection of the immunisation schedule used Freund's Complete Adjuvant (Difco, Detroit) combined with an equal volume of antigen. The second injection, 1 month later, consisted of an equal volume of antigen emulsified in Freund's Incomplete Adjuvant. Groups 1 and 4 were injected each time with 1.0 ml of antigen preparation (average A_{280} 2.0). Groups 2 and 3 received an average of 2.0 ml of the respective antigen preparation (average A_{280} 1.0) at each injection. Concentrations of the respective colloidal antigen preparations used for immunisation were approximated by absorbancy at A_{280}. More exacting measurements were not deemed necessary in view of the relative impurity of the preparations and the linearity of absorbancy to protein concentration. Group 5 received 1.0 ml of Freund's adjuvant at each injection. Injections were given intramuscularly (IM) in opposite thighs.

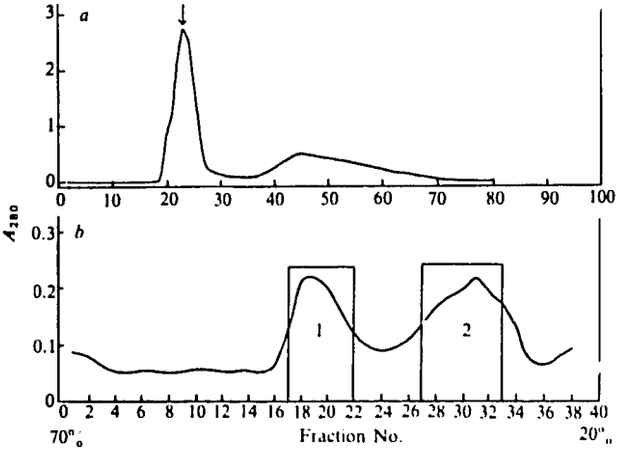

FIG. 1 The first stage of antigen preparation followed the protocol detailed by Schenkel *et al.*[1,5] with the exception that final French Pressure Cell (FPC) effluent was subjected to molecular sieve column chromatography on Bio-Gel A1.5 (Bio-Rad, Richmond, California), rather than on Sephadex G-200. Subsequent fractionation as described in this report focused on the void volume eluate, (arrow, Fig. 1*a*), shown in earlier studies to contain the immunising material, and to be free of detectable host cell contamination[1-5]. Fractionation of the Bio-Gel void volume eluate was accomplished by linear sucrose density gradient centrifugation[6]. The gradients, with terminal concentrations of 20–70%, were prepared by means of a gravity flow apparatus at 4° C. Each cellulose tube contained a total of 29.0 ml. Centrifugation (at 25,000 r.p.m. for 240 min at 4° C) was carried out in the Beckman Model L ultracentrifuge, equipped with a SW 25.1 head (Beckman, Spinco Division, Palo Alto, California). Samples (0.7 ml) were collected by means of a fractionator using needle puncture of the gradient tubes. A typical profile is seen in Fig. 1*b*. Vaccination preparations consisted of respectively pooled peaks 1 and 2. Sample pools were dialysed overnight against PBS (2-3 changes) to remove the sucrose.

TABLE 1 Treatment, survival, and peak parasitemias of vaccinated and control monkey challenged with *Plasmodium knowlesi*.

Treatment	Group No.	Monkey No.	Monkey surviving challenge = +	Peak parasitemia after initial challenge
Bio-Gel void volume		1486	+	<0.5
eluate preparation in	1	1481	+	7.5
Freund's adjuvant		1493	+	2.5
Peak 1 sucrose density		1484	−	0.0
gradient fraction in	2	1489	−	0.5
Freund's adjuvant		1491	+	2.5
Peak 2 sucrose density		1483	−	6.0
gradient fraction in	3	1492	+	<0.5
Freund's adjuvant		1497	+	<0.5
Bio-Gel void volume		1476	−	50.0
eluate-no adjuvant*	4	1477	−	58.0
Freund's adjuvant-		1479	−	24.0
no antigen	5	1480	−	48.0

* In earlier published data[1] the void volume eluate without adjuvant had been shown to be non-protective, thus, this group constituted an additional control.

The challenge inoculum consisted of approximately 2×10^7 *P. knowlesi*-infected red blood cells, given intravenously 1 month after the second antigen injection. The progress of the infection was monitored by percentage parasitaemias, red blood cell counts, and microhaematocrits. (Due to the fulminant nature of the infection, determination of exact parasitemias at death was difficult to obtain. For this reason, the observed parasitemias of unprotected animals may have been, in many cases, artificially low.)

The significance of differences in mortality rates was calculated by Fisher direct *P* significance test.[7]

All surviving monkeys were rechallenged with 1×10^8 parasitised RBC of the homologous *P. knowlesi* strain 3 months after the initial challenge. At that time the red blood cell counts and microhaematocrit values of these animals had returned to prechallenge levels, and no parasites were detectable in peripheral blood smears.

The monitored responses to the initial challenge can be generally categorised into three classes: 6/9 vaccinated animals (Groups 1, 2 and 3) which demonstrated parasitaemias of 0-7.5% and survived; 3/9 vaccinated animals (Groups 1, 2 and 3) which demonstrated similar parasitaemias, but did not survive; and, 4/4 control animals (Groups 4 and 5) which demonstrated extremely high parasitaemias of up to nearly 60% and died.

In Group 1, all three animals survived challenge with the average maximum parasitaemia being 4%. In Group 2, two of three animals died after challenge, although the parasitaemias of all animals in this group were extremely low (average maximum parasitaemia, 1.3%). At necropsy one of the two non-surviving monkeys (1489) showed evidence of a respiratory infection. Two of three monkeys in Group 3 survived challenge. The parasitaemias of all animals were very low and that of the survivors did not exceed 0.5%.

A wide range of indirect evidence[8] suggests that Peak 1 contained primarily the malarial pigment, haemozoin, together with protective antigenic material. Peak 2 seemed to be essentially free of iron, as assessed by atomic absorption

spectrophotometry, and contained protective antigenic material. Thus, it had been anticipated that both sucrose gradient peaks would confer protection against a challenge of *P. knowlesi*.

All animals in Groups 4 and 5 died of a rapidly fulminating infection, reflecting the normal course of the disease in non-protected Rhesus monkeys. The parasitemias and RBC counts of these monkeys were indistinguishable from the numerous non-injected controls used in previous experiments. The lack of protection of animals in Group 4, which received Bio-Gel void volume eluate without adjuvant, corroborated our earlier experiments and those of other investigators[1,9,10], and emphasised the importance of combining the antigen with an effective adjuvant. Group 5 represents a control to detect non-specific resistance induced by Freund's adjuvant alone. No such resistance was observed.

The peak parasitaemias of the nine animals in experimental Groups 1, 2 and 3 were similar, whether or not the animals survived, and were significantly lower than those of the animals in the control Groups 4 and 5 (Table 1). Figure 2 indicates that the course of the anaemia was similar in all animals.

Upon rechallenge none of the surviving monkeys succumbed to the infection and peak parasitaemias were not in excess of 1%.

These results confirm and extend the earlier findings of Schenkel *et al.*[1,5] concerning the feasibility of vaccinating sub-human primates. However, one of the more striking observations concerns the drop in red blood cell profiles following challenge. After a slight initial rise in both RBCs and microhaematocrits, the blood indices dropped dramatically, even in protected groups where parasitaemias remained <5% or were entirely undetected. A similar but smaller decline was observed upon rechallenge of surviving animals.

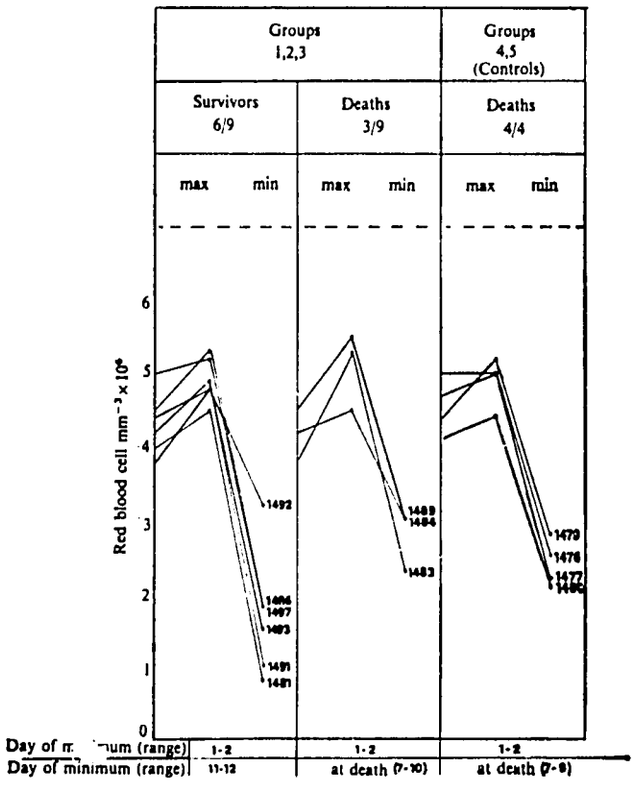

FIG. 2 Maximum and minimum RBC counts following initial challenge. Animals are divided into the three defined classes (vaccinated-surviving; vaccinated-fatality; control).

The aetiology of the anaemia could be postulated as due to some complication of the vaccination process and/or the natural course of the infection, as erythrocyte destruction greater than that predicted from the percentage of cells infected has been well documented[11–18]. Also, certain factors of the present experimental design may be relevant. Unlike previous trials, in this experiment juvenile Rhesus monkeys (1.5 kg weight), rather than adult animals (6 to 7 kg), were used, thus more closely simulating the immune status of children, and the challenge inocula were extremely status of (Analysis of minimum infective dosage in the *P. knowlesi*-Rhesus monkey system has demonstrated that the number of parasitised erythrocytes sufficient to cause death approaches five: L. Schmidt, personal communication.) The contributions of each of these parameters, or combination of parameters, to the observed haemolysis is unkown.

In this study, mortality figures (antigen in Freund's adjuvant versus control groups) become significant to less than 1% only when the results of the preceding vaccination series[1,5] are included. Summation of the data produces a total series of twenty five monkeys. Eleven of seventeen vaccinated animals have survived challenge, while none of the eight control animals have survived ($P < 1\%$).

In summary, we have demonstrated that a significant decrease in parasitaemia and an increase in survival of Rhesus monkeys infected with *P. knowlesi* can be induced by the administration of non-viable sucrose density gradient fractions, in Freund's adjuvant, of the void volume preparation used successfully in earlier studies[1,5].

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