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9. ABSTRACT

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Delayed Dermal Hypersensitivity in Rhesus Monkeys (*Macaca mulatta*) Immunized against *Plasmodium knowlesi**

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Summary. Eighteen Rhesus monkeys were immunized with a fresh or lyophilized antigen preparation obtained from erythrocytes infected with *Plasmodium knowlesi*. Sixteen of these monkeys showed a positive delayed hypersensitivity response when subsequently skin tested with the immunizing antigen. Ten of the 16 positive reactors survived a *P. knowlesi* challenge, the remaining 6 showed several indications of protection. None of the 18 control monkeys gave a positive skin reaction or survived the challenging inoculum. Macro- and microscopical evidence suggests that the dermal reaction is of a delayed type.

Preliminary biochemical analysis of the antigen used in this experiment is included and its significance is discussed. Hematological data is also presented.

Introduction

Intradermal skin testing in malaria has been reported previously. Taliaferro and Bloom (1945) noted an increase in inflammation and rate of phagocytosis at the test site in chronic Rhesus monkeys (*Macaca mulatta*) injected intracutaneously with *Plasmodium knowlesi* infected blood. Makari (1946a, b) tested individuals infected with malaria with an aqueous extract of human malarious blood and observed a maximum skin reaction at 24 h. Phillips et al. (1970) found that Rhesus monkeys suffering from a chronic *P. knowlesi* infection, when challenged subcutaneously with *P. knowlesi* infected cells, gave an immediate type response which was maximal at 4 h. These monkeys also showed an inflammatory response which reached a peak at 24 h.

The ability to vaccinate Rhesus monkeys against *P. knowlesi* has been demonstrated (Freund et al., 1945; Targett and Fulton, 1965; Brown et al., 1970; Schenkel et al., 1973; Simpson et al., 1974; Schenkel et al., 1975; Mitchell et al., 1975).

We attempted to establish an in vivo assay which would enable us to predict, prior to challenge, whether vaccinated monkeys would be protected. If a malarial vaccine suitable for human use is developed, such a test would be useful in determining whether vaccinated individuals would be resistant upon exposure to infection or if re-vaccination is indicated.

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Materials and Methods

Animals. Thirty-six male and female Rhesus monkeys (*M. mulatta*) ranging in weight from 2.5 to 5.4 kg (mean 4.05 S.D. ± 0.81) were divided into 9 groups (Tables 1, 2). All of the animals had been purchased from Primate Imports[1] and were kept under controlled temperature (27° C), and a 12 h light-dark cycle. They were fed Purina Monkey Chow twice a day and allowed to drink water ad lib. from an automatic dispenser. Monkeys were housed individually in cages that complied with U.S. Federal regulations. The waste collecting pans and the rooms where the animals were kept were washed once a day.

Antigen Collection. Antigen was prepared from Rhesus monkey blood infected with the H-strain of *P. knowlesi*, as previously described (Schenkel et al., 1973). Immediately after collection the antigen was lyophilized or quick frozen in dry ice-acetone and stored in liquid N_2. Lyophilized antigen was reconstituted in sterile distilled H_2O immediately before use.

Antigen Analysis. The protein content of the *P. knowlesi* antigen as determined by the method of Lowry et al. (1951) was used as a measure of the amount of antigen used. DNA and RNA were measured by the diphenylamine (Burton, 1955) or the orcinol reaction (Schneider, 1957), respectively. Carbohydrates were measured by the phenol-sulfuric acid reaction (Ashwell, 1957). Phosphorous determinations for phospholipids were carried out by the ascorbic acid method as modified by Chen et al. (1956). The cholesterol content of the antigen was estimated by the Liebermann-Burchard colorimeter reaction (Abell et al., 1952).

Skin Testing. The abdominal area of 34 of the monkeys was shaved at least 24 h before testing. One week before the first vaccination, all monkeys were injected intradermally (ID) in the abdomen with 0.1 ml of phosphate buffered saline (PBS) containing 30 μg of *P. knowlesi* antigen and at a separate site with 0.1 ml of PBS as a control. The test sites were checked at 6, 24, 48 and 72 h.

All animals were tested a second time 3 weeks after the second vaccination, i.e. one week before challenge, and the skin reaction sites examined at intervals of 6, 24, 48 and 72 h. The diameter of the indurated area was measured with a sliding caliper. Reactions with a diameter >5 mm were considered positive.

For light microscope observations, skin test biopsies were taken at 0, 6, 12, 24, 48 and 72 h after injection of antigen and were fixed in Karnovsky's fixative (Karnovsky, 1965) for 5–6 h. Tissues were then rinsed in 0.1 M cacodylate buffer, post-fixed in 2% osmium tetraoxide in cacodylate buffer for 2 h, dehydrated in ethanol and propylene oxide and embedded in Epon 812. One-micron thick sections obtained by sectioning blocks on a Porter-Blum MT2B ultramicrotome were stained with methylene blue in borax and then examined with a Zeiss Photomicroscope III.

Challenge. The H-strain of *P. knowlesi* maintained in liquid N_2 was injected into a normal Rhesus monkey. When this animal showed a patent infection, blood was withdrawn and diluted in sterile PBS. All of the monkeys were then challenged by an intravenous injection of 1 ml of the diluted blood containing $2.5 \cdot 10^5$ parasitized RBC.

The parasitemia was followed by examination of Giemsa stained blood films taken daily for a period of 5 weeks after challenge.

Hematology. Hematological observations were carried out 1–2 times a week during the vaccination procedure and 3 times a week after challenge. Red and white blood cell counts were obtained by the use of a Coulter Counter[2]. Packed cell volume (PCV) readings were obtained by the use of an International Microcapillary centrifuge model MB.[3]

Experimental Protocol. Ten experimental groups are included in this report. The groups are identified by the letters A–I. The monkeys are numbered consecutively 1–36 (Tables 1, 2).

Group A (monkeys 1–4) received no treatment, but was challenged with infected RBC. All of these animals were skin tested. Three monkeys showed tuberculous lesions upon autopsy.

Group B (monkeys 5–10) was brought into the monkey-holding facilities after the tuberculous animals had been removed and the building decontaminated. These monkeys were not

1 34 Munson Street, Port Washington, New York 11050, USA
2 Coulter Electronics, Inc., Hialeah, Fla., USA
3 International Equipment, Needham Heights, Massachusetts, USA

vaccinated but were challenged with infected RBC. Four animals were skin tested, the remaining two were not tested and were used as controls to determine if the antigen introduced during skin testing imparted any protection.

Group C (monkeys 11-14) was injected with PBS emulsified in Freund's Complete Adjuvant (FCA) followed one month later by PBS emulsifed in Freund's Incomplete Adjuvant (FIA). One monkey (# 11) had tuberculous lesions in its lungs. All four animals were skin tested and then challenged with *P. knowlesi* infected RBC.

Group D (monkeys 15-18) was similar to group C except that these monkeys, as those in group B, had not been exposed to tuberculosis (TB).

Groups E–H (monkeys 19-32) received varying amounts of lyophilized *P. knowlesi* antigen (Table 2) incorporated in FCA for the first injection and one month later the same amount of antigen emulsified in FIA. Groups E, F and H had been exposed to tuberculosis since monkeys 19, 22, 23 and 29 had lung and visceral tuberculous lesions. Group G had not been exposed to the TB infection. After being skin tested, each monkey was challenged with 2.5×10^5 infected RBC.

Group I (monkeys 33–36) is similar to groups E–H, except that the antigen was fresh and had not been lyophilized. All four animals had been exposed to TB, but only monkey 33 showed lung and visceral lesions.

Immunization of groups D–I were carried out intramuscularly in the flank.

Statistical Analysis. The data was analysed by an analysis of variance technique (Steel and Torrie, 1960). Bayes least significant difference test (Duncan, 1965) was used for comparing mean pairs only when the F-ratio was significant ($P < 0.05$) in the analysis of variance. Simple correlation coefficients (r) were calculated (Huntsberger, 1967) and only those coefficients with $P < 0.025$ were considered biologically significant.

Results

Vaccination. After challenge with *P. knowlesi*, all monkeys which were not vaccinated with plasmodial antigen (Groups A–D) died of a fulminating infection. No significant difference occurred between the average day of death (8.7 ± 0.95) of uninjected monkeys (Groups A–B) and that (8.4 ± 1.06) of adjuvant controls (Groups C–D) (Table 1).

In the 5 groups of vaccinated animals, 11 of 18 monkeys survived the challenge (Table 2). The 7 animals that died showed some indications of protection. The average day of death (14.3 ± 3.3) of these animals was significantly different ($P < 0.05$) than that of either unvaccinated controls or animals treated with Freund's adjuvant.

Six of the surviving monkeys had parasite counts ranging from 110 to 820 infected RBC per 10,000 RBC, monkeys 22, 28, 30 and 35 had relatively low infections lasting 11, 4, 11 and 13 days, respectively, and monkey 26 showed no parasites. Statistical differences were not significant among the 4 groups receiving varying amounts of antigen. The prepatent period of vaccinated monkeys (7.94 ± 3.6) was significantly longer ($P < 0.05$) than either unvaccinated (4.8 ± 0.9) or Freund's adjuvant controls (4.4 ± 0.5).

We have also confirmed in this experiment our previous findings (Schenkel et al., 1975) that the non-viable antigen can be lyophilized without losing its protective effect. Group I received 1 mg of unlyophilized *P. knowlesi* antigen. Group G received the same amount of antigen after lyophilization and reconstitution. Apart from a significant ($P < 0.05$) difference in prepatent period, no other differences could be detected.

Table 1. Results of skin testing and parasitemia of untreated controls and monkeys immunized with phosphate buffered saline (PBS) emulsified in Freund's complete adjuvant (FCA) and one month later with PBS emulsified in Freund's incomplete adjuvant (FIA). All the monkeys were challenged one month after the second immunization with 2.5×10^5 RBC parasitized with *Plasmodium knowlesi*

Group	Monkey	Treatment	State of health[a]	Skin test results	Outcome after challenge[b]	Pre-patent period in days	Maximum number of parasites/ 10^4 RBC	Day of death[c]
A	1	none	+	negative	D	4	2,930	7
	2	none	+ + +	negative	D	4	2,600	9
	3	none	+ +	negative	D	4	4,000	9
	4	none	-	negative	D	4	1,900	8
					Means:	4	2,857	8.25
B	5	none	--	negative	D	6	3,870	9
	6	none		negative	D	5	4,500	9
	7	none	--	negative	D	4	7,200	8
	8	none	-	negative	D	6	3,200	10
	9	none	-	not done	D	6	2,700	10
	10	none		not done	D	5	5,730	8
					Means:	5.33	4,532	9
Means ± standard deviation of non-vaccinated controls (Groups A-B):						4.8 ± 0.92	3,863 ± 1,604	8.7 ± 0.95
C	11	FCA & FIA	+ + + +	negative	D	4	1,100	7
	12	FCA & FIA		negative	D	4	4,000	8
	13	FCA & FIA		negative	D	4	4,000	8
	14	FCA & FIA		negative	D	4	2,520	10
					Means:	4	2,950	8.25
D	15	FCA & FIA		negative	D	5	5,200	8
	16	FCA & FIA		negative	D	5	3,750	10
	17	FCA & FIA		negative	D	4	2,300	8
	18	FCA & FIA		negative	D	5	3,100	8
					Means:	4.75	3,587	8.50
Means ± standard deviation of adjuvant controls (Groups C-D):						4.4 ± 0.52	3,246 ± 1,268	8.4 ± 1,06

[a] No signs of tuberculosis. + Small (2-3 mm) tuberculous lesions in bronchial lymph nodes. + + Large (-3 mm) tuberculous lesions in bronchial lymph nodes. + + + Tuberculous lesions in lymph nodes in the parenchyma of the lungs. + + + + Tuberculous lesions in lymph nodes, lungs, spleen and liver

[b] D Dead; S Survivor

[c] Day of challenge is day "0"

Table 2. Results of skin testing and parasitemia of Rhesus monkeys immunized with *Plasmodium knowlesi* fresh or lyophilized (lyoph) antigen and challenged with $2.5 \cdot 10^5$ RBC parasitized with *P. knowlesi*. For abbreviations, see footnotes to Table 1

Group	Monkey	Treatment[a]	State of health	Skin test results in mm of induration	Outcome after challenge	Pre-patent period in days	Maximum number of parasites 10^4 RBC (patent period)	Day of death
E	19	250 (lyoph)	+ + + +	3.8	D	7	2,000	11
	20		–	22.0	S	7	220 (13)	
	21		–	13.7	S	5	110 (10)	–
F	22	500 (lyoph)	+ + + +	13.2	S	8	90 (11)	–
	23		+ + +	14.6	D	9	500	17
	24			14.5	D	13	1,450	19
G	25	1,000 (lyoph)	–	14.5	D	11	330	16
	26		–	10.5	S		0	–
	27			7.7	S	8	130 (10)	–
	28		–	6.0	S	19	0.8 (4)	–
H	29	1,500 (lyoph)	+ + +	3.2	S	5	280 (16)	–
	30		–	15.0	S	7	18 (11)	
	31			9.9	D	5	4,700	10
	32			8.4	D	6	3,100	12
I	33	1,000 (fresh)	+ + + +	6.1	S	6	820 (16)	–
	34			15.0	S	8	340 (12)	–
	35			5.7	S	6	62 (13)	–
	36			9.7	D	7	2,100	15
Means ± standard deviation for all vaccinated monkeys Groups E–I						8.05 ± 3.3		14.3 ± 3.4

[a] Expressed as the μg of *P. knowlesi* antigen injected with FCA in the first injection and with FIA for the second injection

Skin Test. All of the monkeys that were skin tested responded negatively prior to treatment. After the vaccination schedule had been completed, all of the control animals (# 1–18) continued to respond negatively to the intradermal injection of *P. knowlesi* antigen. A positive reaction was observed in 16 of the 18 vaccinated monkeys (Table 2). Of these 16 positive reactors, 10 survived challenge and the remaining 6 showed a significantly delayed day of death. There is a positive correlation ($r = 0.80$, $P < 0.025$) between the day of death of the vaccinated animals and the size of the reaction site at 24 h (Fig. 1).

Statistical analysis reveals that the correlation coefficient of vaccination doses and size of the skin reactions is $r = 0.39$. This negative correlation is not statistically significant ($P < 0.35$).

Macroscopically, the test site remained negative for approximately 24 h after the tests were performed. The reaction reached a maximum between 24 and 48 h

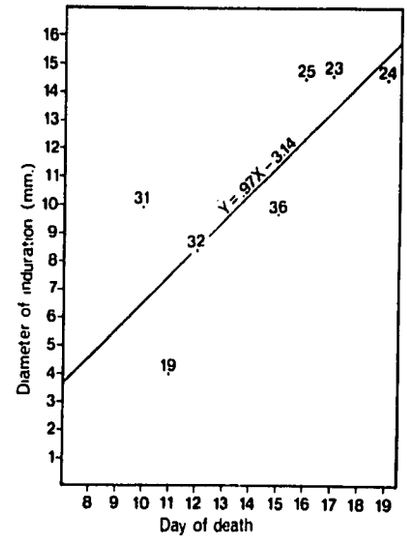

Fig. 1. Relationship between the diameter of induration of the site injected with 30 μg of lyophilized antigen and the day of death of seven immunized monkeys. The average day of death of control animals was 8.6 (range 7–10). Individual monkeys are represented by the number closed to the circles

and, in those animals where no skin biopsies were taken, decreased by 72 h. Erythema and induration were observed in all positive reactions.

Microscopic examinations of skin biopsies taken at 0, 6, 12, 24, 48 and 72 h reveal an increase in infiltrating cells between 0 and 48 h. A maximum number of infiltrating cells was observed at 48 h. Over 70% of the infiltrating cells were mononuclear, only a few polymorphonuclear cells were seen. A detailed microscopical study of the reaction sites will be published elsewhere (Speer et al., 1976a).

Controls consisting of PBS were included in all skin testings. At no time during the observation period was a positive reaction recorded for the control site.

All the results indicate that the dermal hypersensitivity observed was mainly of the delayed type. There was no evidence of Jones-Mote type hypersensitivity in any of the reacting sites. However, at 6 and 12 h there was a slight infiltration of polymorphonuclear leucocytes, particularly neutrophils. Arthus hypersensitivity cannot be absolutely ruled out because no serum transfer experiments were performed. However, because the time course of the reaction does not follow the kinetics of an Arthus reaction and because no edema and little erythema were observed, a typical Arthus reaction was probably not present.

Hematological Observations. Control groups (A–D) showed a decrease in RBC count and PCV (Table 3) which is expected in animals infected with *P. knowlesi*. However, since some of the infected controls were weak and lethargic several days prior to death and restraining them for blood collection might have caused

Table 3. Red blood cell counts (RBC), packed cell volume (PCV) and white blood cell counts (WBC) on the control monkeys prior to challenge and for a 35 day period following challenge. For treatment of different groups consult Table 1

Group	Monkey	Before challenge		After challenge					
		RBC $\times 10^{-6}/mm^3$		RBC $\cdot 10^{-6}/mm^3$		PCV		WBC $\cdot 10^{-3}/mm^3$	
		Normal[a]	6 days after second vaccination[b]	Max.	Min.	Max.	Min.	Max.	Min.
A	1	5.57 ± 0.25	—	5.98	4.46	42	33	26.0	8.0
	2	5.47 ± 0.19	—	5.92	4.33	36	27	13.2	5.3
	3	5.52 ± 0.20	—	5.65	3.88	41	28	8.6	5.4
	4	5.36 ± 0.19	—	5.48	3.56	39	27	15.2	6.6
B	5	6.36 ± 0.45	—	7.05	3.53	47	23	11.0	10.9
	6	5.73 ± 0.62	—	6.10	2.40	42	18	12.7	6.4
	7	6.65 ± 0.56	—	6.85	2.05	46	15	18.8	5.1
	8	5.78 ± 0.56	—	6.10	4.56	30	33	9.1	6.6
	9	5.46 ± 0.29	—	6.30	3.72	40	24	11.3	9.9
	10	6.22 ± 0.33	—	5.80	3.77	41	29	10.9	6.6
C	11	5.77 ± 0.13	5.51	6.15	4.70	42	32	12.2	6.1
	12	5.57 ± 0.21	5.29	6.12	4.83	40	32	16.1	8.6
	13	5.30 ± 0.09	5.15	5.47	4.18	39	30	13.3	7.0
	14	5.34 ± 0.17	5.12	5.29	3.83	38	29	24.2	10.2
D	15	6.07 ± 0.83	6.03	6.06	4.70	40	34	18.3	10.7
	16	5.34 ± 0.39	4.86	5.10	2.92	34	21	16.0	8.6
	17	6.03 ± 0.79	not available	6.17	5.60	38	33	14.3	11.8
	18	5.73 ± 0.52	5.63	5.66	5.01	41	37	14.3	7.3

[a] Mean ± S.D. of at least 4 counts

[b] Groups A & B received no vaccination. Groups C & D received adjuvant alone

traumatization, some of the counts recorded as minimum were taken 2–4 days prior to death.

The white blood cell counts were made on the same blood samples used for the RBC count and PCV so they again denote the same bias noted above. Most of the maximum values for WBC are from the last blood sample taken from the animals which indicates that the WBC count rises with an increase in parasitemia.

The vaccinated groups (E–I) showed a decrease in RBC count and PCV value (Table 4). In those animals surviving the infection, minimum RBC counts and PCV coincided with a peak parasitemia. Soon thereafter they recovered and blood values were back to normal within 30 days after challenge. Monkeys 26, 28 and 30 that showed few or no parasites in their peripheral blood had a small change in blood values which can be attributed to the variation in normal Rhesus monkeys.

The WBC counts in these groups reflect the variation observed in normal Rhesus monkeys. However, the maximum values observed are slightly higher than the values observed in normal animals.

Table 4. Red blood cell counts (RBC), packed cell volume (PCV) and white blood cell counts (WBC) on the experimental monkeys prior to challenge and for a 35 day period following challenge. For treatment of different groups consult Table 2

Group	Monkey	Before challenge		After challenge					
		RBC · 10^{-6}/mm³		RBC · 10^{-6}/mm³		PCV		WBC · 10^{-3}/mm³	
		Normal[a]	6 days after second vaccination	Max.	Min.	Max.	Min.	Max.	Min.
E	19	4.97 ± 0.17	4.61	5.81	4.74	32	25	15.5	7.3
	20	5.56 ± 0.26	5.48	5.95	4.00	42	28	13.1	8.9
	21	5.22 ± 0.25	5.21	5.55	3.03	39	22	25.8	9.0
F	22	5.03 ± 0.21	4.70	6.04	2.63	38	17	15.4	5.9
	23	5.73 ± 0.26	5.97	6.55	4.38	41	27	9.8	4.7
	24	5.63 ± 0.21	5.94	6.19	3.40	40	16	13.2	3.4
G	25	6.00 ± 0.95	5.52	5.68	5.10	36	31	22.2	11.8
	26	5.94 ± 0.72	5.74	6.47	5.43	44	39	20.2	6.4
	27	5.58 ± 0.41	5.88	6.53	4.28	40	28	15.4	5.5
	28	5.06 ± 0.97	5.70	6.46	5.02	41	30	19.2	10.0
H	29	5.79 ± 0.27	5.62	5.68	2.85	37	18	25.5	12.8
	30	5.23 ± 0.12	4.92	5.84	4.48	36	27	14.9	6.0
	31	4.91 ± 0.16	5.01	5.30	2.76	37	20	11.9	7.1
	32	5.36 ± 0.25	4.99	5.93	2.34	45	19	24.0	5.8
I	33	5.30 ± 0.26	5.11	5.89	2.57	39	18	19.4	5.6
	34	5.25 ± 0.28	5.08	5.80	3.01	39	22	13.7	6.0
	35	5.55 ± 0.14	5.53	6.36	4.36	42	30	28.1	12.4
	36	4.96 ± 0.25	4.85	5.21	3.83	31	21	11.9	3.9

[a] Mean ± S.D. of at least 4 counts

Table 5. Biochemical analysis of the French-press *Plasmodium knowlesi* antigen used for immunization

	Amount	# Determinations
Protein	1,000 μg	5
Phospholipids	163.7 ± 3.5 μg	3
Cholesterol	48.9 ± 4.8 μg	5
RNA	61.8 ± 16 μg	4
Carbohydrates	traces	5
DNA	traces	5

Antigen Analysis. Upon biochemical analysis, several different batches of *P. knowlesi* antigen consisted of mostly protein with considerable amounts of cholesterol and phospholipids, moderate amounts of RNA and traces of carbohydrates and DNA (Table 5).

Discussion

Vaccination Effects. In the present study, an attempt was made to establish a lower threshold for the amount of antigen necessary to confer protection against *P. knowlesi* in Rhesus monkeys. However, the lowest dose used in this study (250 μg protein antigen/injection) proved to be protective. Evidently, the lower threshold is below the 250 μg dose used. The protective effect of lyophilized and fresh antigen were similar. Comparable doses of both antigens induced almost identical protection in recipient monkeys (Groups G and I, Table 2). The only difference was a significantly ($P < 0.05$) longer prepatent period in the monkeys receiving the lyophilized antigen over those animals receiving the fresh antigen. This confirms our earlier finding (Schenkel et al., 1975) that *P. knowlesi* antigen can be lyophilized and still retain its protective qualities.

Hematological Studies. Most of the vaccinated animals that recovered from the *P. knowlesi* infection (20, 21, 22, 27, 29, 30, 33, 34 and 35) had a drop in RBC and PCV values, which may be due to the low patent *P. knowlesi* infection observed in these animals. This idea is supported by monkeys 26, 28 and 30, which had no or extremely low parasitemia and showed a slight drop in RBC and PCV values.

Any effect of the *P. knowlesi* antigen as an inducer of anemia can be ruled out by the observation that after the second vaccination the blood counts remained normal until the monkeys were challenged with parasitized blood (Table 4). If the antigen was causing an autoimmune response because of RBC contamination, the second injection of the antigen should bring about an anamnestic response resulting in RBC destruction. Additional evidence that the antigen preparation is not contaminated with host's RBC has been previously reported (Beckwith et al., 1975). The possible contamination of the antigenic material with erythrocyte antigens, has also been tested in subsequent experiments by skin testing the vaccinated monkeys with normal Rhesus RBC ghosts. No positive reactions were ever observed at this test site (unpublished observations).

Skin Testing. From the macro- and microscopic observations, the reaction observed in 16 or the 18 vaccinated animals is probably a delayed type hypersensitivity reaction. This conclusion is based on the following results: 1) The kinetic of the response is similar to that of a typical delayed response in that the reacting sites reach a maximum size at 24 h and remain positive until 48 h. Thereafter, there is a decrease in the size of the reaction area which is minimal at 72 h. 2) More mononuclear cells were present at the reaction site than polymorphonuclear leukocytes and 3) The gross examination of the reaction revealed mostly induration with some erythema but no edema.

This delayed skin response correlated well with the delay in the day of death of those vaccinated monkeys that succumbed to the infection (Fig. 1). However, we could not detect a significant correlation between the size of the skin response of those surviving the challenge and the peak parasitemia reached by these animals. The fact that some of the vaccinated animals succumbed to the infection could be due to the high challenge dose used in these experiments (2.5×10^5).

The protective immune response against malaria parasites has not been satisfactorily elucidated. However, it seems that this response is not exclusively

humoral nor cellular. The fact that most Rhesus monkeys showing delayed dermal hypersensitivity against a lyophilized *P. knowlesi* antigen were protected (either survived or showed a significant delay in the day of death) tends to support the idea that cell mediated immunity (CMI) is an important part of this protective immune response.

Both vaccinated animals that did not react to the intradermal injection of *P. knowlesi* antigen (# 19 and 29) had active tuberculosis (Table 2). Cutaneous anergy to several antigens has been previously reported in patients with TB (Kent and Schwartz, 1967). Therefore, the possibility that cutaneous anergy played a role in the negative response of these animals cannot be ruled out.

Recently Bazaz-Malik (1973) reported that *Mycobacterium tuberculosis* infection increased the resistance of Rhesus monkeys to malaria. These investigators showed that Rhesus monkeys harboring a TB infection could survive a primary challenge with *P. cynomolgi* while comparable non-TB controls died of the infection. Similar groups of monkeys challenged with *P. inui* also demonstrated some resistance of TB positive monkeys over non-TB controls. When TB positive Rhesus were challenged with *P. knowlesi* little or no difference was observed when compared to TB negative controls. The available data from Freund et al. (1964) on TB positive Rhesus challenged with *P. knowlesi* shows no apparent difference between these monkeys and TB negative animals. The presence of TB did not appear to influence the outcome of *P. knowlesi* infection in animals that had been immunized with formalin-killed *P. knowlesi* (Freund et al., 1945). In the present study, we found no difference in the outcome of *P. knowlesi* infection in Rhesus monkeys regardless of the presence or absence of TB.

Antigenic Analysis. The biochemical nature of the protective antigen in our *P. knowlesi* preparations is as yet uncertain. The analysis of this preparation (Table 5) reveals that RNA is present. This nucleic acid is capable of inducing an immune response (Smith and Bigley, 1972; Neiburger et al., 1973). Ribosomal vaccines against *M. tuberculosis* (Youmans and Youmans, 1965), *Salmonella typhimurium* (Johnson, 1972), *Trichomonas foetus* (Oka, 1967), *Leishmania enriettii* (Preston and Dumonde, 1971) have been successful in protecting the experimental animals against the infective agent.

Although the RNA found in our immunizing material could be the protective antigen, electron micrographs show the antigen to consist mostly of irregularly shaped vesicles limited by unit membranes (Speer et al., 1976b). The presence of RBC membrane has been practically ruled out by the studies of Beckwith et al. (1975) who failed to detect phosphatidylserine in the antigen preparation. This phospholipid is present in RBC membrane as 22–14% of the total phospholipid content, however, it is practically absent in the *P. knowlesi* membrane (Rock et al., 1971). It is of interest to point out that lyophilization of membranes appears to alter conformation and change their immunogenicity by altering immunologically active sites, which cannot be restored by rehydration (Nowotny, 1972). The protective immunogen might be a membrane component whose key antigenic determinants are not affected by lyophilization.

It is possible that by varying the dose of antigen used in the skin testing or by lowering the dose of parasites used for challenge, the delayed dermal hypersensitive reaction described in this work could become an effective tool for evaluating the immune state of vaccinated monkeys. These results provide

additional indirect evidence to support the idea of involvement of the CMI system in protection against malaria. Other correlates of CMI need to be investigated in order to confirm and expand our results.

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