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Long-Term Studies on Rhesus Monkeys (*Macaca mulatta*) Immunized Against *Plasmodium knowlesi*

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Studies carried out on four rhesus monkeys (*Macaca mulatta*) that had been vaccinated against *Plasmodium knowlesi* show that the immunized animals were protected against a challenge with a heterologous strain of *P. knowlesi*. This protection was shown to be present even 4 years after the immunization schedule had been completed. The effect could not be attributed to previous infections with the parasite, since four control rhesus monkeys that had recovered from one to four challenges with *P. knowlesi* died when exposed to the heterologous strain. Data obtained from the lymphocyte transformation test and the radioimmunoassay are also presented.

The number of laboratory investigations dealing with vaccination of rhesus monkeys (*Macaca mulatta*) against *Plasmodium knowlesi* has increased in the last 6 years. Successful immunization studies with dead parasites (4), fresh merozoites (7), and nonviable antigen extracted from blood parasites (5, 8, 11) give considerable support to the feasibility of a malaria vaccine. However, there are many questions that remain unanswered. The studies presented in this publication have been directed to the questions of (i) the length of immunity and (ii) the ability of our vaccinating material to protect against heterologous variants and strains of *P. knowlesi*.

When studying the length of immunity against malaria in the rhesus monkey-*P. knowlesi* system, a major consideration is the use of adequate controls. Vaccinated animals that are challenged a relatively short time after vaccination and are used later for the purpose of establishing the long-term effect of the vaccination cannot be compared with normal animals, since the immunized monkeys received not only the antigenic material but also the challenging dose of parasites. An ideal control for this type of experiment would be a monkey that spontaneously recovered from a *P. knowlesi* infection. This is not likely to occur with infections of *P. knowlesi* induced by inoculation with fresh blood. However, when the blood has been kept in liquid N_2 storage, spontaneous recovery occasionally occurs (10).

Over the last 3 years we have had three monkeys recover spontaneously from infections initiated in our laboratory with frozen inoculum. These animals are included in this experiment as controls to assess the longevity of protection observed in the vaccinated monkeys.

Antigenic variation is a well-documented phenomenon that occurs in the *P. knowlesi* infection of rhesus monkeys (2). There is a certain amount of intrastrain cross-immunity in chronically infected monkeys. However, the interstrain cross-immunity observed in similar circumstances is probably less marked or absent (4). For this reason, the monkeys in the experiments presented here were challenged with a strain of *P. knowlesi* different from the strain used for vaccination and for primary challenge.

MATERIALS AND METHODS

Monkeys. Male and female rhesus monkeys were used throughout these studies. The four experimental monkeys (1486, 1481, 1493, and 1491) were immunized as juveniles (1.5 to 2.0 kg). These animals as well as the control monkeys weighed 4 to 6 kg at the time this experiment was carried out.

Parasites. *P. knowlesi* H strain was originally isolated by W. Chin et al. (6) and was sent to us by W. E. Collins (Center for Disease Control, Atlanta, Ga.). Two serologically distinct variants of this strain were used. The H_1 variant was used to prepare the antigen and for the first two challenges of the vaccinated monkeys. Variant H_2 was used for the rest of the challenges involving this strain.

P. knowlesi W strain was obtained from Walter Reed Army Institute of Research, Washington, D. C.

Blood examination. Thick and thin blood smears were examined daily for a minimum period of 3 weeks after infection. The number of parasitized erythrocytes per 10^4 erythrocytes was recorded.

Blood samples were taken four times during the 4-week period and leukocyte and erythrocyte counts

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were performed in a Coulter counter Z_{III} (Coulter Electronics, Inc., Hialeah, Fla.).

Schizont-infected cells agglutination test. The schizont-infected cells agglutination test was carried out as outlined by Brown and Brown (3).

Immunization. The immunization schedule, antigenic material, and other factors concerning immunization have been published elsewhere (9, 11). Briefly, the animals were immunized intramuscularly with a French press antigen preparation emulsified with Freund complete adjuvant. One month later a similar antigenic preparation mixed with Freund incomplete adjuvant was given. Both immunizing doses were prepared from *P. knowlesi* (H strain)-infected blood.

Control monkeys. The three rhesus monkeys that had been exposed to *P. knowlesi* (H strain) and had recovered spontaneously received varying doses of these parasites at different time intervals. A detailed history of each of the monkeys is recorded in Fig. 1.

Monkey 01 had received an inoculation of *P. knowlesi* (H strain) and was cured, after the parasitemia had reached 3%, with daily intramuscular injections of 100 mg of Bactrovet (sulfadimethoxine) (Pitman-Moore, Washington Crossing, N.J.) for 1 week. This animal received no additional challenges or treatment until the rechallenge with the W strain of *P. knowlesi*.

One additional control (2882) had never been exposed to *P. knowlesi*.

Experimental monkeys. The four experimental animals were immunized as juveniles and had been challenged several times with the H strain of *P. knowlesi*. Details of the responses of these animals to these challenges are summarized in Fig. 2.

Heterologous strain challenge. All the monkeys in this experiment were challenged with 10^4 erythrocytes infected with the W strain of *P. knowlesi*. No serological cross-reactivity between the W and H strain could be detected using the schizont-infected cells agglutination test.

Parasite sterility test. To ascertain whether two of the vaccinated monkeys (1481 and 1491) were carrying a subpatent infection, 3 ml of fresh blood was withdrawn from each animal and injected intravenously into a splenectomized uninfected rhesus monkey. Thick and thin blood films were taken daily for at least 2 weeks.

Immunological studies. Serum samples were col-

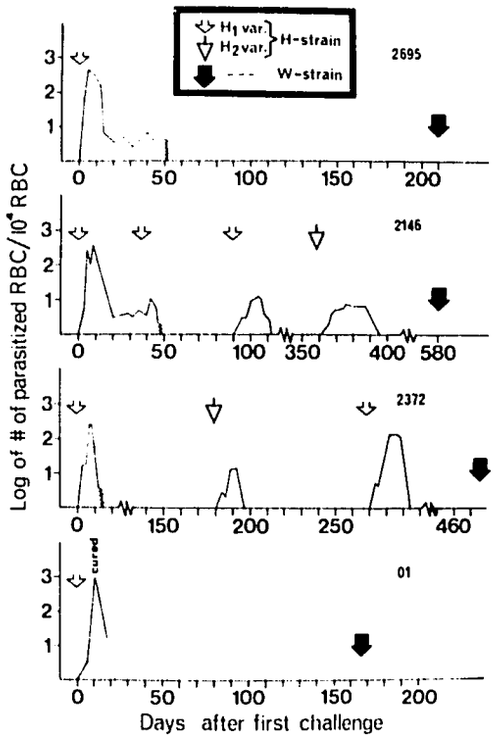

FIG. 1. *Response of the four control monkeys that had been exposed to P. knowlesi. The number of the control monkey appears in the top right-hand corner of each graph. The open arrows represent a challenge with the H_1 variant. Closed arrows represent a challenge with the H_2 variant. The solid arrows represent the time at which the animals were challenged with the W strain. The broken lines in the graphs indicate that the last blood film taken was still showing parasites.*

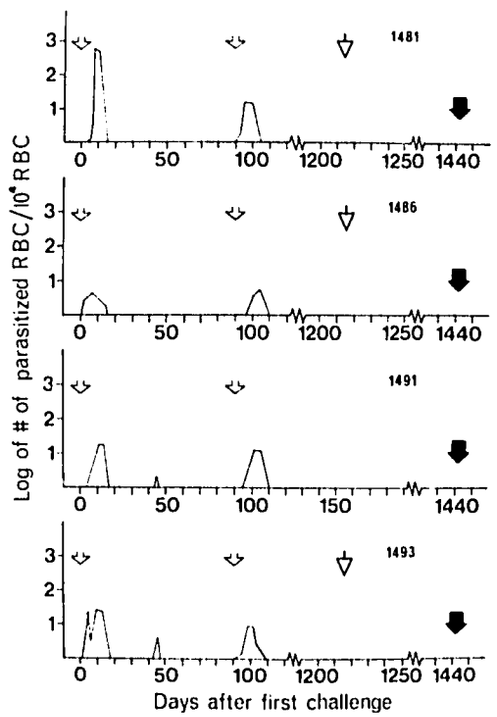

FIG. 2. *Response of the four monkeys that had been immunized against P. knowlesi. The number of the immunized monkey appears in the top right-hand corner of each graph. All these monkeys were challenged for the first time 36 days after the second immunization. For explanation of the symbols, see Fig. 1.*

lected from the animals in this study at the time of the heterologous challenge (W-strain challenge). The specific immunoglobulin content of the serum samples against antigenic preparations of both W- and H-strain origin was assessed by the solid-phase radioimmunoassay (T. Stewart, E. J. Cabrera, R. H. Schenkel, M. L. Barr, and P. H. Silverman, manuscript in preparation). Briefly, the appropriate antigen (either of W- or H-strain origin) was bound to the walls of plastic tubes. The test serum, diluted 1:100, was added and incubated for 1 h. After this period, the test serum was washed out and rabbit immunoglobulin anti-monkey-immunoglobulin G labeled with ^{125}I was added and incubated for 2 h. The radioactivity bound to the walls of the tubes was counted in a Baird-Atomic gamma spectrometer. The results are expressed in specific counts per minute (counts per minute in antigen-coated tubes minus counts per minute in tubes coated with an unrelated antigen).

At the same time that the serum samples were taken, 10 ml of peripheral blood was collected. Mononuclear cells were isolated by the use of lymphocyte separation media (Bionetics Laboratory Products, Kensington, Md.). The isolated cells were washed with Hanks balanced salt solution and cultured in medium 199 (GIBCO, Santa Clara, Calif.) supplemented with 10% decomplemented rhesus serum, L-glutamine (100 mM/ml), 50 μg of streptomycin, and 100 U of penicillin per ml. Approximately 10^6 to 1.3×10^6 mononuclear cells were cultured in the presence or absence of 10 μg of the appropriate *P. knowlesi* antigen (either of W- or H-strain origin). Quadruplicate cultures were prepared and incubated for 5 days. At the end of the incubation period, 2 μCi of [^{3}H]thymidine (specific activity 2 Ci/mmol) (New England Nuclear, Boston, Mass.) was added and the cultures were incubated for an additional 4 h. The cells were harvested by collecting them in glass-fiber filters with the use of a multiple sampling manifold (Millipore, Bedford, Mass.). The cells were then washed two times with cold saline, two times with cold 5% trichloroacetic acid, and once with cold absolute methanol. The dried filters were placed in liquid scintillation counting vials and 5 ml of Aquasol (New England Nuclear, Boston, Mass.) was added. The radioactivity left in the filters was measured in a Beckman LS-230. The results are recorded as blastogenic indexes (counts per minute on stimulated cultures divided by the counts per minute on the nonstimulated cultures).

RESULTS

H-strain challenges. The three animals that recovered spontaneously from challenges with the H strain of *P. knowlesi* showed similar responses (Fig. 1). Monkey 2695 was challenged once with the H_1 variant, and the parasitemia reached 5% on the 6th day postchallenge and then decreased. This animal became chronically infected. Monkey 2146 received three challenge inoculations with the H_1 variant and one with the H_2 variant over a period of 395 days.

Monkey 2372 was infected with the H_1 variant twice and once with the H_2 variant. At no time during the 2- to 3-week period after challenge did the parasitemia of this animal surpass 2.5%.

Monkeys 1481, 1486, 1491, and 1493 were challenged for the first time with 2×10^7 erythrocytes parasitized with *P. knowlesi* (H strain) 1 month after the second vaccination (Fig. 2). All four animals showed a patent infection that lasted approximately 13 days, with a maximum parasitemia of 7.5% reached by monkey 1481. The second challenge consisted of 10^8 parasites of the same strain. At this time all four animals showed a parasitemia below 1%. These initial two challenges were several orders of magnitude higher than the 10^4 dose used in other experiments. In spite of this the vaccinated monkeys were protected. Additional information about the first two challenges is presented in Fig. 2 and has been published elsewhere (11).

Over 1,100 days after the second challenge, monkeys 1493, 1486, and 1481 were inoculated with 10^4 parasites of the H strain but of a heterologous variant (H_2). No parasites were detected in thick smears of peripheral blood from any of the monkeys after this challenge. Normal animals challenged with the same variant showed the typical lethal pattern of *P. knowlesi* infection in the rhesus monkey.

The monkeys were not monitored with blood films between challenges; therefore, the parasite might have relapsed during these time periods. However, blood films taken the day all the animals were challenged with the W strain were negative.

W-strain challenges. The results of the challenge with 10^4 erythrocytes parasitized with the W strain of *P. knowlesi* are summarized in Table 1. All the spontaneously recovered monkeys succumbed to the infection in a manner characteristic of nonprotected animals. A similar pattern was shown by monkey 01. The normal animal (2882) was cured when the parasitemia had reached 1.5% for use in subsequent experiments.

The monkeys (1481, 1486, 1491, and 1493) that had been vaccinated over 4 years previously, showed a delayed prepatent period and a maximum parasitemia well below 1% after inoculation with the W strain of *P. knowlesi* (Fig. 3). The erythrocyte and leukocyte counts remained normal throughout the observation period after this challenge. Observation of these monkeys through the 3-week period after challenge showed a normal consumption of food and water as well as normal behavior.

Three months after the last challenge, monkey 1486 was found dead. This animal died of

TABLE 1. *Response of control and experimental rhesus monkeys to a challenge with 10^4 erythrocytes infected with Plasmodium knowlesi (W strain)*

Monkey no.	Status of monkey	Days since[a]		Prepatent period[b]	Patent period[b]	Maximum parasitemia (parasites/10^4 RBC)[c]	Minimum RBC count (10^{-6}/mm^3)
		First chall.	Last chall.				
2695	SR[d]	210		4	4-8	3,350 (D)[e]	3.28
2146	SR[d]	587	275	7	7-9	3,850 (D)	3.39
2372	SR[d]	475	198	3	3-8	2,050 (D)	5.87
01	Cured	177		3	3-8	3,850 (D)	4.22
2882	Normal			4	4-6[g]	150[g]	5.60
1491	VR[f]	1,442	1,352	6	6-12	36	5.33
1493	VR[f]	1,442	227	8	8-9, 13	1.8	5.58
1486	VR[f]	1,442	227	9	9-10	2.2	5.08
1481	VR[f]	1,442	227	9	9-14	44	5.01

[a] Days elapsed since the first (or last) challenge (chall.) and the challenge described in this table.
[b] Days counted from the day of challenge.
[c] RBC, Erythrocyte.
[d] SR, Spontaneously recovered monkey.
[e] (D), Died from a *P. knowlesi* infection.
[f] VR, Monkey that had been vaccinated and had recovered from a *P. knowlesi* infection.
[g] Sulfadimethoxine treatment began 3 days after patency developed.

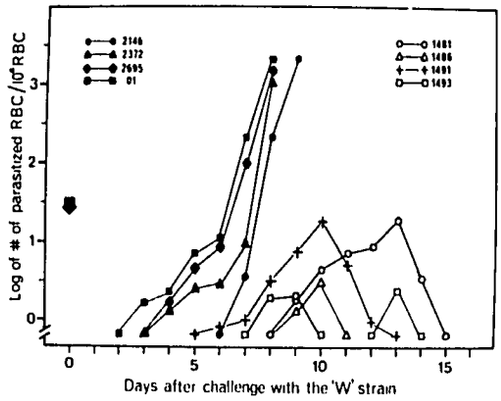

FIG. 3. *Response of the four control monkeys (2146, 2372, 2695, and 01) and the four immunized monkeys (1481, 1486, 1491, and 1493) to a challenge with 10^4 erythrocytes infected with the W strain of P. knowlesi. Solid arrow represents the day of challenge.*

acute gastric dilation (13). Monkeys 1481 and 1491 were tested for sterility of parasites. At this time (3.5 months after challenge with the W strain), the recipient splenectomized monkey injected with 3 ml of blood from 1481 remained negative, whereas a similar test performed with blood from 1491 infected the recipient of this blood.

Radioimmunoassay. The mean specific cpm for the four monkeys that had been vaccinated was 2,423 against the H strain-derived *P. knowlesi* antigen and 1,585 cpm against the W strain for a cross-reactivity of 65%. The unvaccinated monkeys showed much lower specific counts (473 cpm vs. H strain and 136 cpm vs. W strain for a cross-reactivity of 29%).

Lymphocyte transformation. The mean blastogenic index of the vaccinated animals was 9.81 when cells were stimulated by homologous antigen (H strain) and 8.90 when stimulated by the heterologous one. This yields a cross-reactivity of 90%. The unvaccinated controls have blastogenic indices that were not significant.

DISCUSSION

We have demonstrated in this study that the protective action of the antigenic material (8, 9, 11) used to vaccinate rhesus monkeys against *P. knowlesi* is long lasting. The four experimental animals that had been vaccinated as juveniles survived challenge with a heterologous strain of the parasite 4 years and 3 months after the immunization scheduled had been completed. This long-term effect cannot be explained by attributing the recovery of the animals to previous encounters with the parasite, since control monkeys that had recovered from one to four challenges with *P. knowlesi* died when infected with a heterologous strain. However, we cannot rule out the possibility of a synergistic effect between vaccination and natural infection.

The four experimental monkeys survived challenges with a heterologous variant (H_2) and a heterologous strain (W strain). Therefore, we have also shown that our immunizing material probably has an antigen(s) which is shared or cross-reacts with at least two different strains of the parasite. This cross-reactivity was also observed in vitro in immunological reactions. Over 60% of the specific anti-*P. knowlesi*-antigen immunoglobulin G found in

the animals immunized and challenged with the H strain reacted with a similar antigenic preparation from the W strain of *P. knowlesi*. The peripheral lymphocytes obtained from these animals incorporated [^{3}H]thymidine at a high rate whether they were stimulated with antigen obtained from parasites of the H (blastogenic index = 9.81) or the W strain (blastogenic index = 8.90). This degree of cross-reactivity indicated that the French press material has an antigenic site(s) that is common to these two and perhaps to more strains of *P. knowlesi*.

Our immunizing material is obtained from intraerythrocytic stages of the parasite (9); however, other successful immunization studies with the *P. knowlesi*-rhesus monkey system (7) use the short-lived merozoite stage as the antigenic material. Recently, we have determined that there is a great deal of in vitro cross-reactivity between the two antigenic preparations (E. J. Cabrera et al., unpublished data) both at the cellular and the humoral levels. We suggest that the antigenic material capable of interstrain reactivity may be found in several, if not all, of the erythrocytic stages of the parasite. Additional studies of our antigen seem to indicate that the bulk of the material is ultrastructurally (12) and biochemically (1, 5) parasite-derived membrane. These studies implicate the parasitic membrane as a possible site for an intrastrain antigen(s) capable of inducing protection.

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