MALARIA IMMUNIZATION IN RHESUS MONKEYS

A Vaccine Effective Against Both
the Sexual and Asexual Stages of Plasmodium knowlesi

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With malaria a resurging public health problem in much of the tropical world, the search for newer methods to combat this disease has been intensified. Current research is multifaceted, directed toward the development of better means of mosquito control, new chemotherapeutic drugs, and an effective vaccine.

Malaria is caused by a protozoan parasite with a complex life cycle and several different forms and stages (Fig. 1). Infection in the mammalian host is initiated with the injection by mosquitoes of sporozoites that invade hepatic parenchymal cells. Here, each sporozoite can develop into 20,000 or more merozoites; each merozoite, after leaving the liver, is capable of infecting an erythrocyte. Within erythrocytes, merozoites asexually reproduce to form schizonts which in turn produce more merozoites; after cell rupture these then reinvoke other erythrocytes. It is this cycle of cell rupture and reinvasion by asexual parasites that causes the clinical disease.

The sexual cycle of the parasite is equally complex. Some merozoites invade erythrocytes and instead of forming schizonts, develop into male or female sexual forms, the gametocytes. When these are ingested by a blood-feeding mosquito, gametocytes give rise to either sperm-like male microgametes or nonmotile female macrogametes in the gut of the mosquito. These gametes fuse and the resulting ookinetes (zygotes) penetrate through the gut epithelium of the mosquito and produce oocysts. It is the oocyst which ultimately produces the sporozoites found in the salivary gland of the mosquito and which are capable of infecting the vertebrate host to complete the cycle of transmission when the mosquito feeds again.

Malaria parasites are intracellular for most of their existence within the vertebrate host. However, for short periods, different forms of the parasites are extracellular; merozoites are free briefly in the plasma after rupture of the schizont and before erythrocyte reinvasion, sporozoites are free from the time of injection by the mosquito until they invade a liver cell. Gametes are also free from cells in the gut of the mosquito. It is thought that during these brief extracellular periods merozoites, sporozoites, and gametes are vulnerable to immune factors that affect their infectivity, and it is on this vulnerability that anti-merozoite (1), anti-sporozoite (2), and anti-gamete (3) vaccines are based.

Interference with sexual development of the malarial parasite would have an obvious effect on the transmission of the organism from host to host and could act to limit the spread of the disease. Such a scheme for interference with sexual development and transmission has already been demonstrated with the avian malaria, Plasmodium gallinaceum, in chickens (3, 4). After immunization by intravenous injections of X-irradiated or killed sexual parasites, chickens remain susceptible to infection with
parasitized blood or sporozoites, but possess antibodies which prevent the sexual development of the parasite. That is, antibodies produced by the immunized chicken are ingested together with the gametes in the process of feeding and are able to neutralize male gametes and probably female gametes within the gut of the mosquito vector. Consequently, in mosquitoes fed on such immunized chickens fertilization is blocked, oocysts and sporozoites do not develop, and the mosquito is unable to transmit the parasite to other chickens.

Previous studies have shown that gamete immunization in chickens gives no protection against the asexual blood stages of the parasite. That is, such chickens are susceptible to subsequent challenge with sporozoites or infected blood, develop normal parasitemias and gametocytes, and suffer all the ill effects of the disease (3, 4).

We now report the successful immunization of rhesus monkeys with sexual and asexual forms of the simian malaria parasite, *Plasmodium knowlesi*. In contrast to the results in chickens this immunization scheme has a twofold effect: (a) anti-gamete antibodies are produced by the monkey which block the sexual development of the parasite within the mosquito vector; (b) immunized monkeys are protected against asexual parasites and have significantly reduced parasitemias and consequent pathology.

**Materials and Methods**

Rhesus monkeys, *Macaca mulatta*, of both sexes, weighing 3–6 kg, were used in all experiments. Animals were individually caged in a room with constant temperature (24 ± 2°C) and relative humidity (60 ± 5%) with illumination between 6 a.m. and 6 p.m.

Two species of malaria were used in these studies. Two strains of the first species, *P. knowlesi*, one from Malaysia (H strain) (5) and the other from the Philippines (P strain) (6) usually induce fatal infections in rhesus monkeys. The second species of malaria, *P. cynomolgi* (Gombok strain) (7) from Malaysia, produces a less severe nonfatal infection in rhesus monkeys. Both species of these simian malarias will also infect man. The two *P. knowlesi* strains and *P. cynomolgi* produce viable gametocytes in rhesus monkeys (gametocytes make up ñ1–2% of the total parasite population in all cases). Gametocytes of these two species are highly infectious to the *Anopheles balabacensis* mosquitoes used in these studies.

All infections were induced by the injection of parasitized blood from previously infected

![Diagram of the life cycle of *Plasmodium knowlesi* in the rhesus monkey.](image-url)
donor monkeys. The standard inoculum for all infections, including challenge infections of immunized monkeys, was $10^5$ schizonts administered intravenously around 12 noon. The percentage of erythrocytes carrying the various stages of the malaria parasites was determined by examination of Giemsa-stained thin-blood films. Infections of *P. knowlesi* in nonimmune rhesus monkeys, when initiated by the injection of such parasitized blood, are usually rapidly fatal, leading to a daily 5- to 10-fold increase in parasitemia. To prevent the death of these monkeys, a curative regimen of chloroquin was initiated when parasitemias reached $\approx 15$–25%. Chloroquin hydrochloride (Aralen, Winthrop Laboratories, New York, N.Y.) was administered intramuscularly, 20 mg/kg body weight on the first day followed by 10 mg/kg on each of two successive days.

**Antigen Preparation.** *P. knowlesi* gametes were prepared in two ways for inoculation into monkeys; one crude antigen preparation consisted of parasitized blood in which gamete emergence and exflagellation had been promoted, the second preparation consisted primarily of semipurified micro- and macrogametes and trophozoites. This approach to antigen preparation has been shown to be highly effective in producing antigenic material for immunization of chickens with *P. gallinaceum* (3, 4).

Details of the preparation of these antigens are as follows: blood was drawn between 10 p.m. and 1 a.m. from monkeys with parasitemias between 25 and 50% (at this time only gametocytes and trophozoites are present, no schizonts or merozoites are detectable), washed in 10 vol of a special salt solution designed to inhibit gamete emergence (8), then spun at 500 g for 5 min. The packed cells were then resuspended in 2 vol of heat-inactivated (56°C for 30 min) normal rhesus serum for 30 min at room temperature to facilitate the release of both micro- and macrogametes. The first antigen preparation, referred to as exflagellated blood, was simply a concentration of the crude mixture of erythrocytes containing asexual and sexual parasites, unparasitized blood cells, and free gametes.

The second antigen preparation was a partially purified derivative of the first. Here the crude exflagellated blood mixture was first spun at 500 g for 5 min, the pellet was resuspended in the original supernate and spun again for an additional 5 min. We found that this resuspension in the same supernate followed by the second spin was necessary to leave the majority of free gametes suspended in the supernate, which then contained numerous gametes, some asexual parasites, some erythrocytes, and cell debris. This supernate was then spun at 30,000 g for 10 min at room temperature to facilitate the release of both micro- and macrogametes. The first antigen preparation, referred to as exflagellated blood, was simply a concentration of the crude mixture of erythrocytes containing asexual and sexual parasites, unparasitized blood cells, and free gametes.

**Antigen Administration.** Several different methods of immunization were used. First, the crude exflagellated blood preparation was administered to monkeys intravenously at weekly intervals for 4–7 wk. Each individual injection contained $3 \times 10^6$ unparasitized erythrocytes, $1.5 \times 10^6$ trophozoites, and $5 \times 10^5$ micro- and macrogametes. Second, another group of monkeys received the crude preparation intramuscularly in adjuvant, that is, 0.5 ml of the crude mixture was emulsified in 0.5 ml of Freund's complete adjuvant (FCA) (Bactoadjuvant complete, H37 Ra, Difco Laboratories, Detroit, Mich.) and 0.5 ml of the emulsion was injected into each hip. Each monkey received a total of $3 \times 10^5$ unparasitized erythrocytes, $1.5 \times 10^5$ trophozoites, and $5 \times 10^5$ micro and macrogametes. Third, a group of monkeys received the partially purified parasite mixture with FCA or Freund's incomplete adjuvant (ICA, Difco Laboratories) intramuscularly, again, 0.5 ml of the emulsion was injected into each hip. Each monkey received a total of $10^8$–$10^9$ microgametes, with lesser numbers of macrogametes and trophozoites as determined by counting in a hemocytometer. Whereas some received a single injection in this third group of animals, another group of animals received two such injections 7 days apart. As a control for the effect of FCA alone on the course of infection for the latter two experiments, some monkeys received an emulsion of adjuvant and normal serum before challenge.

After inoculation with any of the antigen preparations, monkeys were given a curative regimen of chloroquin starting on the day of immunization. Chloroquin was administered to prevent the development of parasitemia that might arise from the few infectious forms (trophozoites) in the antigen mixture. FCA-treated control monkeys also received 3 days of chloroquin treatment.

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1. Abbreviations used in this paper: FCA, Freund's complete adjuvant; ICA, Freund's incomplete adjuvant.
Challenge Infections and Assays of Immunity In Vivo. Monkeys were challenged with homologous or heterologous strains of *P. knowlesi* at various intervals after immunization. The standard inoculum of 10⁵ schizonts was given intravenously. Parasitemia was monitored by taking daily blood samples that were examined as Giemsa-stained thin smears. Animals in which the challenge infections showed a rapid rise in parasitemia were drug treated when about 15% of the erythrocytes were infected. Self-limited infections in immunized animals were drug terminated 3-4 days after the peak of parasitemia. Thereafter, monkeys were rechallenged with either strain of *P. knowlesi* or with *P. cynomolgi*. In some experiments, the spleen was removed surgically from monkeys before rechallenge.

To determine the effects of the immunization of the host monkey on the sexual development of the parasite, *A. balabacensis* mosquitoes, natural vectors of *P. knowlesi* and *P. cynomolgi* in southeast Asia, were fed on monkeys from the 2nd day of detectable parasitemia to the day of peak parasitemia. Feeding was accomplished by applying caged mosquitoes to the shaved chest or abdomen of immobilized monkeys; mosquitoes readily fed and became engorged with blood through the screening of the cage. All feedings were done between 11 p.m. and 1 a.m. because of *P. knowlesi*’s highly synchronous cycle of infectivity (time when gametocytes are mature) to mosquitoes (9). Mosquitoes were dissected 7 days after feeding and oocysts growing on the gut were stained with 0.5% mercurochrome and counted. Immunity was indicated by a fall in oocyte number on the guts of mosquitoes fed on immunized and infected monkeys as compared to the number of oocytes observed on the guts of mosquitoes that were fed on monkeys that were infected but not immunized.

In Vitro Assays of Immunity. The presence of anti-gamete activity in the serum of immunized monkeys could be demonstrated by two in vitro tests: (a) Gametocyte-containing blood drawn from a *P. knowlesi* infected monkey was washed in saline, resuspended in various sera, and fed to mosquitoes through a feeding device consisting of a membrane-covered, water-jacketed (37°C) chamber (10). Mosquitoes fed through the membrane on the resuspended blood much as they would feed on a monkey. Anti-gamete activity of a test serum was determined by the reduction in numbers of oocytes on the guts of mosquitoes fed on the gametocyte suspension in test serum as compared to the numbers of oocysts developed on the guts of mosquitoes fed on a suspension of similarly infected erythrocytes suspended in normal serum. (b) Blood drawn from a *P. knowlesi* infected monkey was washed, resuspended in test serum, and observed with a phase-contrast microscope. Microgametocytes initiate exflagellation 15-20 min after suspension in serum at room temperature and microgametes show sperm-like activity for over 30 min. In the presence of immune serum, microgametocytes initiate exflagellation but the resultant microgametes cease movement within 2 min.

A more sensitive variant of this second test utilized a preparation of purified microgametes which were then incubated with test serum at room temperature. In the presence of anti-gamete antibodies in the serum, in contrast to what happens in normal serum, movement of microgametes ceases in <30 s.

Both normal and immune serum samples used in both the in vitro tests were inactivated by heating at 56°C for 30 min before use.

Results

Nonimmunized control monkeys show a typical response to schizont challenge and rechallenge with *P. knowlesi*. For example, after such an infection, monkey no. 426 (Table I) developed a rapidly rising parasitemia and was drug treated when its blood parasite count reached 8%. The animal was later splenectomized and challenged again; again it developed a rapidly rising infection and again required chloroquin to prevent death. In intact animals, however, many repeated rechallenges with parasites of the homologous strain usually leads to a self-limiting infection (11). Blood obtained from both primary and secondary infections in intact and splenectomized monkeys is infectious to mosquitoes, that is, mosquitoes fed on these monkeys develop a large numbers of oocysts; oocyst numbers often exceed 100 per mosquito gut and mean oocyst numbers in excess of 300 are not unusual.

**Immunization with Exflagellated Blood Administered Intravenously.** Four rhesus monkeys
TABLE I

Development of P. knowlesi Parasitemia and Infectivity of Gametes as Measured as Oocyst Development on the Guts of Mosquitoes Fed on Normal Monkeys and Monkeys Pretreated with FCA and Chloroquin

| Monkey no. | Parasite strain | Days after first infection with schizonts | Mean no. oocysts per mosquito gut (10 guts per night)* | Nights fed$ |
|------------|----------------|------------------------------------------|-----------------------------------------------|-------------|
| Untreated control monkeys | | | | |
| 426 | H | 42§ | 12 | 5 |
| | H | 42§ | 480 | 2 |
| | P | 93 | 22§ | 2 |
| | H | 160 | 5§ | 2 |
| 346 | H | 40§ | 63 | 4 |
| | H | 49 | 12 | 3 |
| | P | 133 | 29§ | 3 |
| | H | 190 | 7 | 5 |
| 258 | H | 16§ | 480 | 2 |
| | H | 50 | 6 | 6 |
| | P | 99 | 5 | 3 |
| P. cynomolgi | | | | |
| 182 | 3 | 8.5 | 5 |
| | 565 | 5 | 2 | 4 |
| 108 | P | 38§ | 460 | 3 |
| | H | 88§ | 450 | 1 |
| 918 | H | 28§ | 400 | 3 |
| | P | 56 | 16§ | 3 |
| | H | 18§ | 70 | 2 |
| Monkeys pretreated with FCA and chloroquin+ | | | | |
| 420 | H | 44¶ | 31§ | 29 | 3 |
| | H | 129¶ | 15§ | 350 | 2 |
| | P | 510 | 19§ | 28 | 3 |
| 622 | H | 38¶ | 18§ | 170 | 4 |
| 625 | H | 38¶ | 9§ | 75 | 4 |

* Mean oocyst counts above 100 per gut are estimates.
† Mosquitoes fed on infected monkeys nightly from the second night of patency to the night of peak parasitemia or chloroquin treatment.
§ Monkey treated with 3 days of chloroquin starting when parasitemia reached the level indicated.
¶ Monkey splenectomized 7 days before rechallenge on day indicated.
§§ Monkey pretreated with 0.5ml FCA emulsified with 0.5 ml rhesus serum intramuscularly followed by 3 days of chloroquin, and later challenged on day indicated.

Received from 4 to 7 weekly intravenous injections of the crude exflagellated blood mixture without adjuvant (Table II). When challenged, all four monkeys developed severe infections and required drug treatment. Moreover, all these infected monkeys were highly infectious to biting mosquitoes. There was no evidence, therefore, of an anti-gamete effect or an effect on asexual parasites induced by the intravenous administration of this crude antigen preparation.

Immunization with Exflagellated Blood Administered Intramuscularly in FCA. Four monkeys were immunized with a single intramuscular inoculation of the crude exflagellated blood mixture emulsified in FCA (Table III). After challenge, three of four animals developed low levels of parasitemia, whereas the fourth required drug treatment. More significantly, the first three monkeys completely failed to infect mosquitoes (no oocysts developed), whereas the fourth produced only 13 oocysts in a
TABLE II
The Effect of Intravenous Immunization with Exflagellated Blood Parasitized with P. knowlesi (H strain) on the Severity of Asexual Parasitemia and Infectivity of Gametes as Measured as Oocyst Development on the Guts of Mosquitoes Fed on these Monkeys

| Monkey no. | No. of weekly immunizations | Challenge (days after immunization) | Maximum parasitemia | Mean no. oocysts per mosquito gut (10 guts/night) | Nights fed |
|------------|----------------------------|-------------------------------------|---------------------|---------------------------------------------|------------|
| 808        | 7                          | 26                                  | 34*                 | 250                                         | 3          |
| 142        | 5                          | 26                                  | 23*                 | 460                                         | 3          |
| 146        | 4                          | 36                                  | 9*                  | 16                                          | 2          |
| 147        | 4                          | 36                                  | 20*                 | 180                                         | 3          |

* Monkey treated with chloroquin starting when parasitemia reached the level indicated.

TABLE III
The Effect of a Single Intramuscular Immunization with Exflagellated Blood Parasitized with P. knowlesi in FCA on the Severity of Asexual Parasitemia and Infectivity of Gametes as Measured as Oocyst Development on the Guts of Mosquitoes Fed on these Monkeys

| Monkey no. | Antigen strain | Challenge infection | Maximum parasitemia | Mean no. oocysts per mosquito gut (10 guts/night) | Nights fed |
|------------|----------------|---------------------|---------------------|---------------------------------------------|------------|
| 537        | H              | 51                  | 1.3                 | 0                                          | 5          |
| 144        | P              | 18                  | 14‡                 | 0.05                                       | 2          |
| 440        | H              | 18                  | 1.1                 | 0                                          | 4          |
| 435        | H              | 33‡                 | 0.3                 | 0                                          | 4          |
| 281        | H              | 13                  | 0                   | 0                                          | 6          |

* Monkey splenectomized 7 days before rechallenge day indicated.
‡ Monkey treated with chloroquin starting when parasitemia reached the level indicated.

total of 40 mosquitoes. Nevertheless, all four asexual infections produced gametocytes in numbers that would ordinarily be sufficient to insure good mosquito infections; furthermore exflagellation was noted in the parasitized blood of all four monkeys.

When animals were immunized with FCA alone all developed rapidly rising parasitemias and all were highly infectious to mosquitoes (Table I). Immunization with FCA alone therefore gave no protection against either the asexual or the sexual parasites.

Immunization with Semipurified Gametes in FCA. The results of immunization with the semipurified gamete/trophozoite antigen were more dramatic. 13 monkeys received varying concentrations of the partially purified mixture emulsified in FCA (Table IV). Nine monkeys were challenged with homologous H-strain schizonts; none
developed a serious infection and none required drug treatment. Although gametocytes were less numerous because of low asexual parasitemias, exflagellation was noted in the blood of most of these monkeys. However, all failed to infect mosquitoes after test feeding (no oocysts developed).

To determine the specificity of the immunity produced, two animals (712 and 732) were immunized with semipurified H strain parasites of *P. knowlesi* in FCA and were then challenged with P-strain schizonts (Table IV). Both animals developed rapidly rising infections requiring drug treatment. However, neither monkey was infectious to mosquitoes that had fed on them. When the experiment was reversed the results were somewhat different. Two monkeys (736 and 63) immunized with P-strain parasites and challenged with heterologous H-strain parasites showed immunity against both the sexual and asexual stages.

To test for cross-protective immunity between two diverse malaria species, two monkeys immunized with semipurified parasites of *P. knowlesi* in FCA and previously challenged with *P. knowlesi* (no. 114 and 442, Table IV) and a control nonimmunized monkey (no. 298, Table I) were challenged with blood stages of *P. cynomolgi*. All developed parasitemias typical of *P. cynomolgi* infections and all were infectious to *A. balabacensis* mosquitoes as judged by number of oocysts developed. Thus no cross-species immunity developed.

Immunization using Freund's incomplete adjuvant (ICA) was also found to be effective, although less so than the complete adjuvant in immunizing against challenge with *P. knowlesi*. Four monkeys received the semi-purified antigen emulsified in ICA (Table V). Two were challenged with the homologous strain of schizonts and two with heterologous schizonts. All developed rapidly rising infections and were drug treated; nevertheless, two monkeys showed complete inability to infect feeding mosquitoes whereas the others showed only a low level of infectivity.

One monkey was immunized with a single inoculation of $10^6$ partially purified *P. knowlesi* parasites that had been frozen at $-10^\circ$C for 48 h before emulsification with FCA. After challenge with schizonts 40 days later, its parasitemia did not rise above 5% and it also failed to infect mosquitoes over 5 days of feeding.

*In Vitro Correlations of Transmission Blocking Immunity.* This was measured in two ways. First, sera from all 17 monkeys immunized intramuscularly with antigen in FCA and 4 with antigen plus ICA blocked infectivity of gametocytes fed to mosquitoes through a membrane. That is, no oocysts developed in these mosquitoes whereas mosquitoes fed on the same parasites in normal serum were infected. Second, serum from these same immunized monkeys also immobilized microgametes within minutes; normal serum had no such effect.

Cross-protection between different *P. knowlesi* strains as observed after challenge, was confirmed in vitro by the above tests; sera from monkeys immunized with either P or H gametes showed complete transmission-blocking and microgamete immobilizing actively against both homologous and heterologous parasite strains. However immune serum from these monkeys had no effect in vitro against the sexual stages of *P. cynomolgi*.

Sera from monkeys immunized intravenously (Table II) and repeatedly infected control monkeys (Table I) showed no in vivo transmission-blocking activity and were negative in both in vitro tests.

Finally, to demonstrate that gametocytes from immunized monkeys remained potentially infectious, parasitized blood from such monkeys was drawn, washed, and
### Table IV

The Effect of One or Two Intramuscular Immunizations with Semipurified *P. knowlesi* Gametes in FCA on the Severity of Asexual Parasitemia and Infectivity of Gametes as Measured as Oocyst Development on the Guts of Mosquitoes Fed on these Monkeys

| Monkey no. | Immunization schedule | Challenge infections | Mean no. oocysts per mosquito gut (10 guts/night) | Nights fed |
|------------|-----------------------|----------------------|-----------------------------------------------|------------|
|            | Total dosage microgametes | No. of injections | Parasite strain | Days after immunization | Parasite strain | Maximum parasitemia |                          |
| 533        | $1 \times 10^7$        | 1                    | H               | 21        | H                   | 0.1           | 0                          | 2                      |
|            |                       |                     |                 |                       | 66*                 | 31‡           | 0                          | 3                      |
|            |                       |                     |                 |                       | 114                  | 6‡            | 0                          | 2                      |
|            |                       |                     |                 |                       | 217                  | 8             | 0.04                      | 7                      |
|            |                       |                     |                 |                       | 495                  | 15‡           | 0                          | 3                      |
|            |                       |                     |                 |                       | 600                  | 11‡           | 0.03                      | 3                      |
| 434        | $1 \times 10^7$        | 1                    | H               | 22        | H                   | 5             | 0                          | 4                      |
|            |                       |                     |                 |                       | 67                   | 2             | 0                          | 4                      |
|            |                       |                     |                 |                       | 160                  | 5             | 0                          | 8                      |
|            |                       |                     |                 |                       | 280                  | 0.3           | 0                          | 3                      |
|            |                       |                     |                 |                       | 560                  | 0             | no feeding                 |                          |
|            |                       |                     |                 |                       | 685                  | 0             | no feeding                 |                          |
| 442        | $1 \times 10^7$        | 1                    | H               | 44        | H                   | 0.1           | 0                          | 1                      |
|            |                       |                     |                 |                       | 93*                  | 14‡           | 0                          | 3                      |
|            |                       |                     |                 |                       | 178                  | 12            | 4                          | 4                      |
|            |                       |                     |                 |                       | 518                  | 8‡            | 0                          | 4                      |
|            |                       |                     |                 |                       | 635                  | 26‡           | 0                          | 3                      |
| 429        | $1 \times 10^7$        | 1                    | H               | 44        | H                   | 1.1           | 0                          | 5                      |
|            |                       |                     |                 |                       | 93*                  | 43‡           | 0                          | 6                      |
|            |                       |                     |                 |                       | 178                  | 40‡           | 0                          | 3                      |
|            |                       |                     |                 |                       | 470                  | 8‡            | 0                          | 5                      |
|            |                       |                     |                 |                       | 590                  | 51‡           | 0                          | 6                      |
| 712        | $2 \times 10^6$        | 1                    | H               | 37        | P                   | 25‡           | 0                          | 4                      |
|            |                       |                     |                 |                       | 106*                 | 10‡           | 0                          | 2                      |
|            |                       |                     |                 |                       | 310                  | 34‡           | 0.07                      | 5                      |
| 732        | $2 \times 10^6$        | 1                    | H               | 37        | P                   | 22‡           | 0                          | 3                      |
|            |                       |                     |                 |                       | 106*                 | 7‡            | 0                          | 2                      |
|            |                       |                     |                 |                       | 355                  | 21‡           | 0                          | 2                      |
| 736        | $2 \times 10^6$        | 1                    | P               | 35        | H                   | 6             | 0                          | 9                      |
|            |                       |                     |                 |                       | 101*                 | 8‡            | 0                          | 2                      |
|            |                       |                     |                 |                       | 360                  | 50‡           | 0                          | 3                      |
| 63         | $2 \times 10^6$        | 1                    | P               | 35        | H                   | 0.7           | 0                          | 4                      |
|            |                       |                     |                 |                       | 115                  | 4             | 0                          | 5                      |
|            |                       |                     |                 |                       | 380                  | 5             | 0                          | 3                      |
|            |                       |                     |                 |                       | 465                  | 21‡           | 0                          | 3                      |
| 117        | $2 \times 10^6$        | 2                    | H               | 35        | H                   | 0.5           | 0                          | 2                      |
|            |                       |                     |                 |                       | 91                   | 20‡           | 0.03                      | 3                      |
|            |                       |                     |                 |                       | 455                  | 21‡           | 3                          | 3                      |
| 147        | $2 \times 10^6$        | 2                    | H               | 35        | H                   | 0.4           | 0                          | 1                      |
|            |                       |                     |                 |                       | 91                   | 16‡           | 1.4                       | 3                      |
|            |                       |                     |                 |                       | 171                  | 10            | 5                          | 4                      |
|            |                       |                     |                 |                       | 435                  | 12‡           | 26                         | 3                      |
### Table IV Continued

| Monkey no. | Total dosage microgametes | No. of injections | Days after immunization | Challenge infections | Maximum oocysts per mosquito gut (10 guts/night) | Mean no. nights fed |
|------------|---------------------------|------------------|-------------------------|---------------------|-------------------------------------------------|-------------------|
| 114        | $2 \times 10^5$           | 2                | H                       | H                   | 0.2                                            | 0                 |
|            |                           |                  |                         |                     | 0                                              | 2                 |
| 119        |                           |                  |                         |                     | 0.4                                            | 6                 |
| 150        |                           |                  |                         |                     | 0.1                                            | 5                 |
| 176        |                           |                  |                         |                     | 0.2                                            | 0                 |
| 462        | $2 \times 10^6$           | 2                | H                       | H                   | 0.1                                            | 3                 |
| 474        | $1 \times 10^6$           | 1                | H                       | H                   | 17                                             | 0                 |
| 119        |                           |                  |                         |                     | 37‡                                            | 0                 |
| 310        |                           |                  |                         |                     | 10‡                                            | 0                 |
|            |                           |                  |                         |                     | 0.5                                            | 2                 |

* Monkey splenectomized 7 days before rechallenge day indicated.
‡ Monkey treated with chloroquin starting when parasitemia reached the level indicated.

### Table V

| Immunization schedule | Challenge infections | Mean no. oocysts per mosquito gut (10 guts/night) |
|-----------------------|----------------------|---------------------------------------------------|
| Monkey no.            | Total dosage microgametes | No. of injections | Days after immunization | Parasite strain | Maximum parasitemia | Nights fed |
| 2786                  | $2 \times 10^6$       | 1                  | P   | 35    | H   | 27*   | 0     | 3     |
| 479                   | $2 \times 10^6$       | 1                  | P   | 35    | H   | 20*   | 4     | 3     |
| 506                   | $2 \times 10^6$       | 2                  | H   | 44    | H   | 24*   | 0     | 4     |
| 507                   | $2 \times 10^6$       | 2                  | H   | 44    | H   | 32*   | 0.3   | 3     |

* Monkey treated with chloroquin starting when parasitemia reached the level indicated.

Resuspended in autologous serum which had been taken either before or after immunization (Table VI). All monkeys in this experiment had been splenectomized before rechallenge. Consequently, they developed rapidly rising asexual parasitemia and produced large number of gametocytes. In spite of these high gametocytomias, mosquitoes fed directly on these monkeys were not infected; no oocysts developed. Moreover, mosquitoes fed through membranes on these gametocytes resuspended in autologous postimmunization serum also failed to develop oocysts. However, gametocytes from these same monkeys resuspended in their preimmunization autologous serum were infectious to mosquitoes as determined by oocyst development. Thus, as long as gametocytes in these immune monkeys remained within erythrocyte membranes, they were unaffected by the presence of anti-gamete antibodies in the suspending medium.
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Table VI

| Monkey no. | Parasitemia | Direct feeding† | Resuspended in autologous serum* |
|------------|-------------|----------------|-------------------------------|
|            |             |                | “Normal” | “Immune” |
| 533        | 31          | 0              | 39       | 0       |
| 442        | 14          | 0              | 23       | 0       |
| 429        | 45          | 0              | 34       | 0       |
| 462        | 37          | 0              | 48       | 0       |

* Mosquitoes fed on infected blood through a membrane.
† Mosquitoes fed directly on infected monkey.

Table VII

Summary of Effects in Rhesus Monkeys of Various Immunization Schemes with P. knowlesi Gamete Antigens on the Course of Asexual Parasitemia and Infectivity to Mosquitoes

| Immunization          | Challenge strain | Course of asexual infection* | Infectivity to mosquitoes‡ |
|-----------------------|------------------|------------------------------|-----------------------------|
| Nonimmunized control  | Homologous       | Usually fatal                | High                        |
| Antigen alone intravenously | Homologous       | Usually fatal                | High                        |
| Antigen + FCA intramuscularly | Homologous       | Parasitemia usually <1%     | None                        |
| Antigen + ICA intramuscularly | Heterologous     | Usually fatal§               | None                        |
|                       |                  | Usually fatal                | Minimal or none             |

* Measured as percent parasitized erythrocytes. In uncontrolled infections, parasitemia rises rapidly and results in death of the monkey unless drug treated.
† Measured as oocyst development on the guts of mosquitoes fed on these monkeys.
§ Two monkeys immunized with P. knowlesi gametes and challenged with a heterologous strain controlled their asexual parasitemias; the reciprocal immunization and challenge resulted in a rapidly rising uncontrolled infection requiring treatment.

Discussion

In the present study, monkeys have been immunized with one or two injections of an antigen mixture consisting primarily of microgametes with lesser numbers of macrogametes and asexual trophozoites emulsified in FCA. The resulting antibodies block the sexual development of the malaria parasite within the gut of the mosquito vector. These mosquitoes therefore, cannot transmit malaria. In addition to this effect on the sexual phase of the malaria parasite, there was a marked suppression of the asexual reproductive phase of the malaria parasite in the monkey (Table VII).

The above results should be contrasted with the previous studies of immunity to
the malaria of chickens (*P. gallinaceum*). Chickens required several intravenous injections of a *P. gallinaceum* antigen mixture without adjuvant to establish transmission blocking immunity; monkeys developed anti-gamete transmission blocking immunity after a single intramuscular dose of the *P. knowlesi* antigen preparation in FCA, whereas several intravenous injections of the crude *P. knowlesi* antigen mixture failed to immunize. Finally, in the immunized chicken no protection against the asexual phase of the disease was observed.

To return to the present study in monkeys we will now examine in more detail the mechanism of suppression of both the sexual and asexual phases of the malaria parasite which is observed after successful immunization.

Immunized monkeys produce low but adequate numbers of normal gametocytes; these gametocytes give rise to gametes that are potentially infectious to mosquitoes. However, in the presence of ingested antibody in the gut of the mosquito, fertilization is prevented as judged by the fact that oocysts do not develop. Several mechanisms might explain such gamete inactivation. First, microgametes could be agglutinated in the mosquito gut and would therefore be unable to fertilize the macrogamete. Our results demonstrate that in vitro, in the presence of serum from immunized monkeys, microgamete immobilization is observed. Second, the macrogametes could be coated with antibody which would block fertilization. Finally antibody and complement could lyse the gametes in the mosquito gut. Indeed, in vitro studies have demonstrated that microgametes and macrogametes lyse minutes after immobilization in the presence of immune serum with complement (R. W. Gwadz, unpublished observations).

Anti-gamete antibodies appear to be species, but not strain specific. Thus, monkeys immunized with *P. knowlesi* antigens were not protected against *P. cynomolgi* challenge. However, immunization with parasites of one *P. knowlesi* strain provided protection against the sexual stages of an antigenically different strain of the same malaria. Although the antigen mixture used in this study appears to induce immunity against both sexual and asexual parasites, there is no evidence of immunity against gametes being induced by immunization with sporozoite or merozoite vaccines. That is, preliminary studies in our laboratory (R. W. Gwadz, unpublished observations) have shown that monkeys immunized with *P. knowlesi* sporozoites or merozoites and which are resistant to challenge by these stages have no circulating antigamete antibodies as determined by the various in vitro tests. One of the difficulties in studies of the immune response to malaria parasites is that there is no detailed information as to the chemical nature of the surface antigens of various stages, or whether different stages of the malaria parasite bear the same, over-lapping, or entirely different sets of antigens.

How the gamete/trophozoite antigen mixture induces immunity against asexual parasites is unclear. Although asexual schizonts and merozoites are effective antigens for the suppression of asexual infection (11, 12, 13), because of *P. knowlesi* synchrony neither stage was present in our antigen mixture. Whether the small numbers of trophozoites present in our antigen preparation were responsible for suppression of asexual parasitemia or contributed to the anti-gamete response remains to be determined. However, the antigenicity of pure trophozoite preparations has not, to our knowledge, been tested.

It appears that an anti-gamete response (antibodies produced which can block the sexual cycle in the mosquito) can be induced in the monkey more easily than factors
blocking the asexual cycle. That is, when monkeys are immunized with a less effective combination, the gamete/trophozoite mixture in ICA, the sexual cycle in the mosquito is blocked, but the asexual cycle in the monkey is unaffected.

Prospects for in vitro mass production of gametes of *P. falciparum* for initial testing or eventual vaccine production have been enhanced by recent reports of continuous malaria culture (14) and the controlled production of gametes from such cultures (15). To date, however, the factors governing gametogenesis in culture are unknown, and the gametocytes formed in vitro have so far not been able to infect mosquitoes. Eventually it may be possible to identify and synthesize dominant gamete antigens which might be used for immunization. The requirement for FCA in this immunization scheme presents a serious obstacle to the development of a gamete-based vaccine for human use. Studies to evaluate other adjuvants more suitable for use in humans are currently underway.

Finally, the efficacy of immunization against the sexual stages of a human malaria such as *P. falciparum* remains to be determined. However, a vaccine effective as a single injection capable of interrupting transmission from man to man whereas at the same time reducing the severity of the disease in the infected individual offers a new approach to the control of one of the major diseases affecting man.

Summary

Rhesus monkeys were immunized with a preparation of *Plasmodium knowlesi* parasites containing principally microgametes with lesser numbers of macrogametes and asexual trophozoites. The antigen mixture was emulsified in Freund's complete adjuvant (FCA) and administered intramuscularly. After one or two inoculations of from $10^5$ to $10^7$ microgametes in FCA, monkeys showed high levels of circulating anti-gamete antibodies as demonstrated by various in vitro microgamete immobilization or transmission blocking tests. After challenge with *P. knowlesi*, immunized monkeys developed low level asexual parasitemias and were not infectious to feeding mosquitoes as measured by growth of the parasite on the mosquito gut. Control monkeys developed rapidly rising, usually fatal infections and were highly infectious to mosquitoes.

Anti-gamete antibodies appear to neutralize the sexual parasites and prevent mosquito infection within the gut of the recently fed mosquito vector. Suppression of asexual parasitemia in immunized monkeys may be due to the presence of asexual trophozoites in the antigen mixture or to antigens common to both sexual and asexual stages of the parasite.

A vaccine effective as a single injection capable of interrupting malaria transmission from man to man whereas reducing the severity of the disease in infected individuals offers a new approach to the control of one of the major diseases affecting man.

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