AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D. C. 20523
BIBLIOGRAPHIC INPUT SHEET

FOR AID USE ONLY
Batch 69

1. SUBJECT CLASSIFICATION

A. PRIMARY
Health

NS00-0000-0000

B. SECONDARY
Tropical diseases

2. TITLE AND SUBTITLE
Malaria immunity and vaccination; final research report,1966-1972

3. AUTHOR(S)
(101) Ill.Univ. Dept.of Zoology

4. DOCUMENT DATE
1972

5. NUMBER OF PAGES
44p.

6. ARC NUMBER
ARC

7. REFERENCE ORGANIZATION NAME AND ADDRESS
Ill.

8. SUPPLEMENTARY NOTES (Sponsoring Organization, Publishers, Availability)
(Research summary)

9. ABSTRACT

10. CONTROL NUMBER
PN-AAE-567

11. PRICE OF DOCUMENT

12. DESCRIPTORS
Immunization
Malaria

13. PROJECT NUMBER

14. CONTRACT NUMBER
CSD-1432 Res.

15. TYPE OF DOCUMENT

AID 590-1 (4-74)

CSD- 1432 Reg.
Ill.

AGENCY FOR INTERNATIONAL DEVELOPMENT

CONTRACT NO. AID/csd-1432

MALARIA IMMUNITY AND VACCINATION

UNIVERSITY OF ILLINOIS

DEPARTMENT OF ZOOLOGY

URBANA, ILLINOIS

PAUL H. SILVERMAN, DIRECTOR

FINAL REPORT

1966-1972

TABLE OF CONTENTS

	Page No.
GENERAL BACKGROUND	1
STATEMENT OF PROJECT OBJECTIVES AS STATED IN CONTRACT	2
ACCOMPLISHMENTS	6
TROPHOZOITE ANTIGENS	6
MOSQUITO STAGE ANTIGENS	11
IN VITRO CULTURE STUDIES	20
OTHER ACCOMPLISHMENTS	23
SUMMARY AND OUTLOOK	26
PUBLICATIONS	Appendix 1
FIGURES AND LEGENDS	Appendix 2

FINAL RESEARCH REPORT

GENERAL BACKGROUND

As a result of preliminary discussions in late 1965 and early 1966 between Dr. Paul H. Silverman, then Professor and Head of the Department of Zoology, University of Illinois, Urbana, Illinois, and the Office of Technical Cooperation and Research and the Malaria Eradication Branch, A.I.D., the project supported by this contract was proposed in February, 1966 and subsequently funded, initiating July 1, 1966.

The purpose of the project is to develop a vaccine for use in the prevention of human malaria. A practical immunizing technique would have great significance in assuring and maintaining malaria eradication in the A.I.D.-assisted worldwide program. Over 1.8 billion people live in the originally malarious areas of the world, and the disease remains an important deterrent to social and economic progress in the lesser developed countries. A.I.D. is committed to support of the worldwide malaria eradication program which has successfully eliminated or reduced malaria in most operational areas, thus far reaching about 1.4 billion of the affected population.

Difficult problems have arisen in some areas due to resistance of the mosquito vectors to residual insecticides or resistance of the malaria parasites to synthetic antimalarial drugs. While malaria foci remain in these refractory areas, an enormous investment in time, manpower, funds and materials is seriously threatened. A malaria vaccine would be extremely useful in combatting malaria in such areas, as well as in providing another potent weapon to accelerate and offer increased assurance of malaria eradication. The protection of malaria-freed areas against introduction of

the disease would be much advantaged, as well as the protection of people who must live and work in areas which are not covered or not responsive to conventional protective measures with insecticides and drugs.

STATEMENT OF PROJECT OBJECTIVES AS STATED IN CONTRACT

The General Statement of Work of AID Contract 1432 is as follows:

A. The general objective of this contract shall be the development of a vaccine for use in the prevention and treatment of human malaria. The contractor shall investigate and develop techniques for the preparation and testing of antigens of mammalian malaria using rodent malaria parasites, anopheline mosquito vectors and rodent hosts as an experimental system. The extension of this work to primate malaria will be undertaken as soon as justified by progress.

At the time that the above objectives were written (February, 1966) there existed much doubt as to whether it was biologically feasible to vaccinate against malaria. Although some preliminary work, primarily with avian malaria, had been published in the 1940's which suggested the possibility of a malaria vaccine, this evidence was largely negated by the well publicized failure of a large-scale attempt during World War II (involving almost 200 POW's and other human volunteers) to test a crude malaria (*Plasmodium vivax*) vaccine. The failure of that unfortunate WW II attempt has played a major role in convincing malariologists that vaccination against malaria was "impossible". Because of this background of doubts, the primary objective of the Illinois project was to determine whether it was biologically possible to vaccinate against malaria using non-viable antigens, i.e. a dead vaccine.

The complex nature of malaria immunity involving a parasite which undergoes metamorphosis into distinctive stages during infection of the definitive (mammalian) host, as well as the rapidly changing forms that

occur in the insect vector, suggested that a broad spectrum approach be adopted in investigating various means of applying prophylactic immunization to the interruption of the malaria life cycle. The specific approaches which were proposed are outlined under the Specific Performance section of the Statement of Work.

B. Specific Performances

In carrying out the objectives set forth above the contractor shall:

1. Develop techniques for preparation of antigens from the following:
   a. Sporozoites (infective forms inoculated by the mosquito)
   b. Trophozoites (infective forms found in the blood of mammalian host)
   c. Extractions of mosquito tissues
2. Compare the immunogenicity of the sporozoite and trophozoite antigen preparations.
3. Observe effects on vector efficiency by blood meals containing mosquito antibodies, to test the postulate that mosquito antibodies in the blood of the mammalian host will inhibit the development of the malaria parasite in the mosquito.
4. Characterize all antigens by serologic and biochemical methods.
5. Investigate means to control and standardize antigen production from all sources.

C. Provided that encouraging protectivity is induced in laboratory rodents by some of the immunizing procedures, and upon the mutual agreement of the parties hereto, primate hosts will be investigated in similar manner.

ACCOMPLISHMENTS

The achievements of the group during the past six years are summarized below in accordance with the Specific Performance objectives listed above.

1.a Develop techniques for preparation of antigens from sporozoites (infective forms inoculated by the mosquito).

The production of sporozoites is dependent upon a thriving colony of mosquitoes susceptible to malaria. *Anopheles stephensi* was selected as the

primary experimental species, following comparative studies of A. quadrimaculatus, A. albimanus and A. atroparous as vectors of the rodent malaria, Plasmodium berghei. A variety of factors affecting mosquito rearing and survival of adult infected mosquitoes were investigated. These factors included numerous larval culture conditions, genetic variations, insecticide contamination, sources of blood meals, virus and protozoan parasite infections. The results of these investigations have resulted in significant contributions to the field (Appendix 1:1,3,27,28,29,38,39). Among the contributions has been the development of a method to control a microsporidian infection which previous workers have encountered and found it necessary to destroy all mosquitoes and re-establish the colonies from other sources after extensive fumigation and sterilization of the insectaries (Appendix 1:3). The microsporidian parasite has been identified as a species new to science (and named in honor of Dr. Alger, Nosema algerae) with a wide insect host range suggesting the possibility for use as a biological control agent (Appendix 1:3, 39).

In brief summary, the project achieved production of more than adequate numbers of infected mosquitoes for transmission of malaria and for the production of sporozoite antigen used in the immunogenicity studies described below. The production method requires laborious manual micro-dissection which, although adequate for experimental purposes, does not represent a practical means for large-scale production.

1.b Develop techniques for preparation of antigens from trophozoites (infective forms found in the blood of the mammalian host).

Initial investigations with various trophozoite antigen preparation techniques (utilizing the rodent malaria, Plasmodium berghei) led to the adoption of a basic method originally developed by a Walter Reed group in

their search for a specific complement-fixing malaria antigen. The method involves mechanical disruption under controlled pressure using the French pressure cell and a motor driven hydraulic press. Red blood cells are preferentially fragmented releasing the malaria parasites, apparently with unaltered morphology as determined by light microscopy or by ultrastructural studies (Appendix 1: 24). After release of the parasites by low pressure (800-1000 p.s.i.) treatment, the plasmodia are washed and sedimented to remove intact red and white host cells, hemoglobin and red cell stroma. Finally, the host-free parasites are subjected to high pressure treatment (18,000 p.s.i.) and the resultant effluent sedimented by high speed centrifugation (30,000 xg). The supernatant fluid is then subjected to molecular sieve chromatography. Much of the initial chromatography used Sephadex G-200 at 4°C (Appendix 1:20) but more recently BioGel A1.5 has been found to be equally satisfactory and with the added advantage of a much quicker separation time. With these and other molecular sieve materials, the protective antigen has been consistently found in the void volume eluate (Appendix 1:13, 15, 20; Appendix 2: Fig. 1).

The basic method outlined above has been used for preparation of trophozoite antigens from simian (P. knowlesi) and avian (P. gallinaceum) malaria, in addition to P. berghei. Although there remains many unresolved problems concerning variations in quantitative yields of antigen, as well as questions about contamination with some host materials, the procedure regularly provides a material which has been found to have vaccine properties.

1.c Develop techniques for preparation of antigens from extractions of mosquito tissues.

Antigenic preparations of various mosquitoes tissues, as well as extracts of homogenized whole mosquitoes have been prepared. Mosquito antigens from

midgut, thorax and salivary gland tissues have been extracted and both soluble and insoluble components tested for immunologic activity. When available, some of the soluble antigenic preparations have been eluted through molecular chromatographic sieves for further refinement. The methods for preparation of the various mosquito antigens are very similar to those described in 1.a for the preparation of the sporozoite antigen. These methods, as indicated in 1.a, are laborious and time consuming and during the course of the investigations exercised severe constraints on the size and number of experimental replications undertaken. Nevertheless, adequate antigenic material was produced to enable preliminary data to be gathered that supported the conclusion that mosquito antigens could contribute to an antimalaria prophylactic immunization procedure. This evidence, described below, has provided considerable impetus for the development of an insect *in vitro* tissue culture unit.

2. Compare the immunogenicity of the sporozoite and trophozoite antigen preparations.

TROPHOZOITE ANTIGENS

Rodent Model Systems

The first malaria vaccine model developed with trophozoite antigens utilized the highly inbred A/J mouse (obtained from Jackson Laboratories, Bar Harbor, Maine). It was eventually found that this mouse developed a high level of protection against *P. berghei* infection following a *single* intraperitoneal injection of trophozoite antigenic material 6 to 14 weeks before challenge (Appendix 1:13). *P. berghei* strains NK65 or NYU-2 were used as either protective or infective material with no apparent differences between homologous or heterologous combinations. Subsequently, it was

found that other strains of mice could be used as vaccine models. The inbred DDS mouse (obtained from A. R. Schmidt, Co., Madison, Wisconsin) and the outbred Carworth CF_1 (obtained from Carworth Farms, Inc., Portage, Michigan) were found to respond to vaccination with partially purified trophozoite antigens. Indeed, the DDS mouse, because of its higher mortality and apparent lack of age resistance, seems to offer a better host-model system than the A/J mouse (Appendix 2: Figs. 2,3).

Immunized mice develop low level parasitemias and mild anemias which begin to resolve by the first to third week post challenge. Resolution is followed by occasional slight recrudescences in a few mice for a number of weeks. Various tests (e.g. xenodiagnosis and splenectomy) indicate that the induced resistance results in sterile immunity.

Two characteristics of the model system appear important to the reproducibility of the vaccine effect. First, the immunizing injection and the challenge inoculation must be given by the intraperitoneal route. Multiple injections and the adjuvants so far tested (e.g. Freund's, Alginate, polynucleotides, aluminum hydroxide) do not seem to enhance this immune response. Second, the time interval between initial immunizing injection and challenge inoculation must be no less than 6 weeks and preferably 8 weeks or longer for optimal immunity to develop.

The nature of the mouse resistance response appears to depend, at least in part, upon cell-mediated immune mechanisms (Appendix 1:37). This is partially demonstrated by the action of antithymus serum (ATS) which when administered at the time of vaccination, inhibited the development of resistance. Mice receiving vaccine material alone or vaccine alone followed by ATS at the time of challenge with *P. berghei*, showed transient low parasitemias, mild anemias and rapidly resolving disease characteristic of

protected animals. In contrast, those mice receiving ATS at the time of vaccination, or ATS alone or not treated, developed severe protracted parasitemias and anemias characteristic of unprotected animals.

Numerous important quantitative and qualitative parameters remain to be ascertained concerning the mouse vaccine model system. The resistance and susceptibility characteristics of other strains of mice, the quantitative and qualitative characteristics of the antigen, the relative merit of various routes of immunization and schedules require examination.

Of considerable interest is the finding that different strains of *P. berghei* cross-protect. This is true whether partially purified antigen is used or heat inactivated intact infected red blood cells (Appendix 1:14, 21). On the other hand, *P. gallinaceum* material will not protect mice against *P. berghei*.

In spite of the current limitations of the mouse model system, it has proved invaluable in detecting immunogenic components prepared from trophozoite material. Another finding of considerable interest is the observation that mice vaccinated with trophozoite antigen are resistant to infection with viable sporozoites.

Primate Model System

P. knowlesi infection in Rhesus monkeys (*Macaca mulatta*) is a virulent disease resulting in almost 100% mortality 7-14 days after inoculation. Fresh inocula of parasitized blood from infected monkeys usually cause death within 7-9 days, whereas stabilate material (stored at -90°C or liquid nitrogen) require 12-14 days before death ensues.

The *P. knowlesi*-Rhesus monkey system is of particular interest since early workers demonstrated in the 1940's that vaccination with killed *intact*

trophozoite stages mixed with Freund's complete adjuvant stimulated resistance to homologous infection with viable P. knowlesi.

Preliminary trials at Illinois with a partially purified P. knowlesi trophozoite antigen preparation mixed with Freund's complete adjuvant indicated that protection could be stimulated with our material against infection. This is evidenced by slight transient parasitemias and survival of protected monkeys in contrast to the 100% mortality of unprotected animals. Protection occurred in only those monkeys which were vaccinated with Freund's complete adjuvant-antigen mixture. Antigen or Freund's adjuvant alone were non-protective, as were antigen preparations mixed with aluminum hydroxide or sodium alginate (Appendix 1:19).

The preliminary trial described above with partially purified P. knowlesi trophozoite antigen, utilized a lengthy immunizing schedule and multiple routes of injection, as well as a lengthy delay after immunization and before challenge. These conditions, although useful in order to establish the feasibility of vaccination, resulted in an unwieldy primate model system. To determine whether a simpler and less time-consuming immunizing schedule could be applied, an experiment was performed in which monkeys were given only two injections at a 4 week interval of Freund's adjuvanted trophozoite malaria antigen and challenged 3 weeks later.

Three out of 5 vaccinated monkeys survived a normally lethal challenge infection with P. knowlesi. Two of the monkeys never showed any parasites in their blood, and their hematocrit and red blood cell (RBC) counts remained normal. One monkey demonstrated a peak parasitemia of 2.6%. Although its RBC and hematocrit dropped to approximately 20% of normal, the animal survived and eventually showed a normal blood picture with no parasites in

the blood. Interestingly, the parasitemia fluctuated between 1-2% for 5 days, before parasites could no longer be seen in the blood. This is highly unusual in a P. knowlesi infection, which normally kills the animal within 3-5 days after the appearance of parasites. In addition, the parasitemia always rises rapidly after the first appearance of parasites, and it is not unusual to observe a peak parasitemia in excess of 90%.

Of the two vaccinated monkeys that died, one showed a peak parasitemia of only 5% and apparently cleared the infection since no parasites were seen in the blood 2 days before it died. In spite of the low malaria parasitemia, the red blood cell count and hematocrit dropped to 20% of normal and on the day of death a blood slide revealed a very heavy bacterial infection which was thought to be the primary cause of death. The other monkey which died had been vaccinated with heat-treated antigen and the vaccine appears to have been inactivated by the treatment. The second monkey died shortly after the untreated control animal. Subsequent (8 weeks later) rechallenge of the monkeys which had no or slight parasitemia demonstrated solid immunity. No observable parasitemia occurred while a control monkey died from fulminating malaria after inoculation with parasites from the same challenging inoculum.

These results, which have since been replicated (Appendix 2: Figs. 4,5), confirm our earlier trials with partially purified P. knowlesi antigen and indicate that successful immunization can be established with only two injections and that immunity capable of resisting a fatal infection is present by the seventh week after the initial immunizing injection. This "model" reduces substantially the time span required to test active immunizing substances in primates. Of considerable interest is the fact

that the protection effects are achieved with antigen preparations different from the challenge infection. These results suggest that the serologic differences reported to occur after each schizogony are not due to significant changes in the protective antigen which appears to be stable from infection to infection.

The opportunity afforded by the availability of a primate-malaria vaccine system which responds to a nonviable partially purified antigen lends encouragement to the hope that further isolation and characterization of the active antigen can be achieved and that a successful replacement for Freund's complete adjuvant suitable for human use may be found.

MOSQUITO-STAGE ANTIGENS

Mouse Protection Tests

Until very recently, studies utilizing sporozoites as antigenic material to induce protection against malaria have been relatively scarce compared to those employing the antigens of the blood stages. Workers in the 1940's demonstrated considerable protection in chickens immunized with partially inactivated sporozoites against challenging homologous sporozoites of the avian malaria, *Plasmodium gallinaceum*. More recently (1967-69), workers at New York University injected mice with viable x-irradiated sporozoites of *P. berghei*. The immunized mice were challenged with 2,000 viable sporozoites 2 weeks following the last injection and were found to be resistant. Work carried out by the Illinois group included both (viable) irradiated and inactivated sporozoites confirmed the immunogenicity of this material which stimulated an excellent resistance to sporozoite infection. In addition, however, we have found that certain mosquito antigens are almost equally effective in stimulating a protective response (Appendix 1: 9).

In the following experiments carried out in our laboratory, 4-5 week old A/J inbred female mice were used and randomly grouped according to weight at the beginning of the immunizing schedule. Mice were vaccinated intraperitoneally with inactivated P. berghei NK65 sporozoites or other mosquito material and challenged intraperitoneally with 2,000 viable sporozoites 14 days following the last injection. All mice were examined every two days following challenge for the presence of parasites and those which had positive blood films eventually died. No adjuvants were used in any of these experiments. Untreated and treated control mice were selected in the same manner as the vaccinated mice and were all from the same original batch.

As one of the control groups of mice, a group was regularly included which was injected with a quantity of salivary glands from normal uninfected female Anopheles stephensi comparable to the amount of salivary gland material associated with the manually dissected sporozoite preparations. Typical of the results obtained are the data illustrated in (Appendix 2: Fig. 7) utilizing sporozoites which were inactivated by repeated freeze-thawing. Very similar results were obtained when the sporozoites were heat-inactivated at 42°C for 60 minutes (Appendix 2: Fig. 8). When the quantity of sporozoites was reduced from 450,000/injection to 25,000/injection and the amount of normal salivary gland material comparably reduced, the protective effect of the sporozoite antigen remained high but the salivary gland stimulation was lessened (Appendix 2: Fig. 8).

Subsequently, it was ascertained that six injections of 400-500 μg protein material from normal salivary glands obtained from uninfected A. stephensi females were capable of stimulating a significant resistance

to sporozoite infection and some resistance was still apparent 19 weeks after the final immunizing injection. These findings, together with observations on relative spleen weights between sporozoite and salivary gland-immunized and non-immunized mice (no significant differences), support the view that the protection is specific and related to some common antigen shared by sporozoites and A. stephensi salivary glands. Investigation of the immunogenicity of other organs or tissues of A. stephensi have revealed that similar effects can be obtained with midgut material, extracts of the mosquito head (without salivary glands) and extracts of whole mosquitoes. These results are supported by serologic studies which demonstrated cross reactions between mosquito and sporozoite antigens. Other attempts to find nonspecific protective antigens in other insects (housefly and cockroach) have been negative.

The fact that certain mosquito tissues contain an antigen that has vaccine qualities against sporozoite infection adds impetus to the effort to develop a means of large-scale in vitro culture production of mosquito tissues.

3. Observe effects on vector efficiency by blood meals containing mosquito antibodies, to test the postulate that mosquito antibodies in the blood of the mammalian host will inhibit the development of the malaria parasite in the mosquito.

During the past six years work was undertaken to test the postulate that antimosquito antibodies in the blood of the mammalian host will inhibit the development of the malaria parasite in the mosquito and thus interfere with transmission of the disease. Several approaches were utilized involving Anopheles stephensi as the vector and rabbits, hamsters or mice as the mammal.

Rabbit Studies

The first approach involved the use of rabbits immunized with three different mosquito antigen preparations. The first antigen preparation consisted of a saline extract of ground whole mosquitoes and the insoluble components were used as the second antigen. A third type of antigen was obtained by dissecting the midguts (stomachs) of female A. stephensi and grinding them in a glass homogenizer with 0.85% saline. A series of 5 to 8 injections, each representing 2.0 to 3.4 mg protein was used to immunize rabbits. Replicate treatments for each antigen as well as appropriate control rabbits were provided. A series of seven mosquito feeding experiments were carried out on immunized and control rabbits and observations made for 21 days on the mosquitoes which had ingested blood. The results indicated that the mosquitoes which fed on rabbits immunized with midgut antigen died at a greater rate than those which fed on the other experimental or control rabbits (Appendix 1:4,10).

The reason for the enhanced death rate of mosquitoes fed on rabbits immunized with midgut antigen has been suggested by electron microscopic studies carried out with such mosquitoes and compared with those fed on non-immunized rabbits. Briefly, these studies indicate that the anti-mosquito-stomach antibody has in some way interfered with the ability of the midgut cells to synthesize the digestive enzymes which are responsible for breaking down the blood clot which forms soon after a blood meal. This anti-enzyme activity literally causes the mosquito to starve because it cannot digest the protein. A concomitant development is that the early ookinete (infective) stages in the blood clot may not be released to infect the mosquito. The net result of such action might well be to interfere with malaria transmission.

To determine whether anti-mosquito antibody could interfere with malaria

transmission required a new host system (the rabbit, a lagomorph, cannot be infected with rodent malaria). The hamster was selected because it is susceptible to Plasmodium berghei and provides a good source of gametocytes in the blood stages. In addition, mice were also used as a host system, but they are not as efficient as hamsters in gametocyte production. The source of mosquito antigens and the vector for transmission was Anopheles stephensi. Altogether, several hundred hamsters and mice and several hundred thousand mosquitoes were used for the studies briefly summarized below.

In general, the results of these repeated efforts were inconclusive because of technical problems associated with the toxicity to vaccinated rodents of the non-sterile mosquito antigens and the apparent non-specific adverse effects of rodent blood on the mosquito.

Hamster Studies

In a sequence of experiments, groups of hamsters were immunized with a series of intramuscular injections consisting of 1.5 to 3 mg protein prepared from mosquito mid-guts. Because of the non-sterile nature of the antigen, the injected hamsters developed abscesses which required treatment with antibiotics. Attempts to sterilize the antigen were frustrated by the need to include particulate matter, since previous experience suggested that the solubilized components were not immunogenic. Ultrafiltration and ultracentrifugation methods for sterilization could not be used because these techniques concentrated both the bacteria and the particulate antigens. Prolonged exposure (i.e. more than 9 hours) of the antigen to ultraviolet treatment was partially successful, but the antigenic material itself seemed toxic to the rodents. Many of the animals

died during the immunizing process and those that survived were undoubtedly in a weakened condition which adversely affected their ability to respond immunologically.

Of those animals that survived and which were used as sources of blood meals for mosquitoes, the resultant effects on the mosquitoes were not significantly different between those that were fed on immunized or non-immunized hosts. Cumulative ogives based on mosquito deaths showed relatively high mortalities (in some cases as high as 100%) by the 10th day post-feeding. This high death rate contrasts strongly with our normal experience which, for sporozoite production, regularly results in 80-90% survival of mosquitoes for 18 days. This contrast suggests that the mosquitoes used in the experiments on anti-midgut antibody were sub-normal and may not have reflected a true phenomenon. Since the original rabbit-mosquito experiments, additional improvements in mosquito rearing were made and very likely account for the increased survival rate.

Of the several hamster experiments, one included an approach which combined the prior feeding of mosquitoes on an immunized rabbit followed by feeding on a _P. berghei_ infected hamster (multiple feeding by female mosquitoes has been observed in both anophelines and aedes and this method obviated the necessity of immunizing hamsters). The results of these trials are suggestive of transmission interference. A reduction of 80% in sporozoite production appears to have been achieved based on the number of sporozoites recovered from all infected mosquitoes. While these results are encouraging, it is difficult to make unequivocal claims until more reproducibility is achieved. On the other hand, if the rabbit antibody prevented oocyst formation, this may account for the lower number of

mosquitoes found to be infected in the group fed on the immunized rabbit as compared to the control and the effect may be greater than indicated. Other experiments utilizing rabbit anti-mosquito-midgut-serum indicated that the rabbit antibody did not inhibit exflagellation so that it is assumed that the effect of antibody is produced after ookinete formation.

Mouse Studies

Because of the high mortality in vaccinated hamsters, the mouse was used for a number of additional studies. Some of the antigens used for immunization were treated with ultraviolet irradiation and better survival of mice was achieved as compared to the hamsters. The attempts to test the effects of mice immunized with mosquito antigens on transmission of P. berghei were frustrated by failure to achieve satisfactory feeding and survival of mosquitoes. Only 20-50% of the mosquitoes could be induced to feed on mice, even after exposure for 3 consecutive days (in contrast, nearly 100% of the mosquitoes fed on a rabbit or hamster in 30-40 minutes). Of those mosquitoes which did take a blood meal from the mice, 80-100% were dead by day 15 and in many cases as early as day 10. As previously indicated, this may well be due to the condition of the batches of mosquitoes used in those experiments. On the other hand, there is no question that the mouse is a less attractive animal than either the rabbit or the hamster to A. stephensi.

It may well be that the rodent is a poor model to use in this type of investigation. Nevertheless, a good deal is known about the rodent model system and steady improvements in methods and infection reproducibility have been achieved. Another factor of considerable importance is the method of antigen preparation. With the developments in aseptic rearing and in vitro

tissue cultivation, adequate amounts of sterile A. stephensi antigens can be expected to be available. The sterilely produced mosquito antigens may well eliminate or reduce the anaphylactic and hypersensitivity-inducing toxicity of the antigens produced from the conventionally-reared mosquitoes.

4. Characterize all antigens by serologic and biochemical methods.

Work on the serological and biochemical characterization of functionally protective antigens has been impeded by the complexity of the antigens and the difficulty of relating particular antigenic components to resistance-inducing activities. The major efforts of the group over the past six years has been devoted to establishing vaccine model systems which demonstrate unequivocally that prophylactic immunization is feasible. Nevertheless, considerable work on serological tests and purification and isolation of active antigens was initiated.

The first protective antigen available for purification was that obtained from trophozoite material by the French pressure cell method. As indicated above, this antigen is found in the void volume eluate resulting from the chromatographic column of Sephadex G-200 or from BioGel A1.5. The antigen is a complex mixture and contains parasite material including hemazoin, a degradation product of host hemoglobin. Very likely, it also contains some host products such as red and white cell contaminants. A further serious limiting factor is that all attempts to eliminate the particulate matter from the "partially purified plasmodial fraction" also eliminated the vaccine. Since most biochemical purification techniques depend upon soluble material, this insolubility restricted application of

most chemical separation methods. Efforts to solubilize the antigen included preliminary chemical treatment to remove the insoluble hemozoin to which the soluble active antigen might well be attached. Also attempted, with some preliminary success, has been the application of sucrose gradient techniques which has resulted in an apparent hemozoin-free and active antigen preparation. A variety of solvents have been tested and some evidence that soluble lipoprotein components with antigenic activity has been obtained. Another promising approach is the use of very high pressure to reduce further the particulate material obtained after passage through the French pressure cell. Pressures of up to 40,000 p.s.i. are now possible, which more than doubles the previous treatment pressures.

The use of most serologic tests, also depends on the use of a soluble antigen, although some recent tests which are designed for particulate antigens have become available. Specific antisera have been prepared against the complex antigens, but poor or little correlation with resistance has been observed. In large part this may be explained by the observations that the nature of the immune response to trophozoite stages is at least partially cell mediated in nature. The use of immunological tests which are thought to detect cell mediated immune reactions have proved to be inconsistent. Once again, this might well have been due to the insolubility of the antigen.

In summary, the problem of biochemical and serological characterization is dependent upon further progress towards purification of the active antigen. Since each step in the purification procedure must be monitored by protection testing to determine whether biological activity remains, progress has been slow. However, with availability of model vaccine systems

it is possible to envisage that substantial "breakthroughs" in this area can be made in the near future.

5. Investigate means to control and standardize antigen production from all sources.

IN VITRO CULTURE STUDIES

The demonstration that vaccination with sporozoite and trophozoite antigens stimulate resistance to malaria has sharply vocused attention on the need to develop large scale antigen production methods. It is generally agreed that *in vitro* cultivation techniques offer the best possibility of breaking through the antigen production bottleneck. Towards this end, efforts were undertaken in 1969 to develop an *in vitro* cultivation unit. Initial work was concerned with insect tissue cultivation and in 1972 efforts to culture trophozoite stages were begun.

As a preliminary to insect tissue cultivation, the production of germ-free *A. stephensi* mosquitoes was undertaken to provide a source of (1) primary cell cultures as a potential source of sporozoite-like antigens, and (2) tissues, cells and organs for the development of ookinete, oocyst and sporozoite stages in an aseptic antibiotic-free environment.

New apparatus, methods and media have been devised to produce healthy active *A. stephensi* adults from surface sterilized eggs originally obtained from conventially reared mosquitoes. A technique for blood-feeding asceptically-reared adult female mosquitoes has now made it possible to carry through the entire life-cycle under bacteria-free conditions. The newly hatched germ-free first instar larvae are successfully reared to the adult stages in a time period slightly shorter (2-3 days) than that required under our conventional insectary conditions (Appendix 1:30, 31, 32, 35, 36). This accomplishment is the first report of successfully

reared aseptic anopheline mosquitoes.

Primary Mosquito Cells and Tissues.

Long-term primary cultures of cells in which fragments of newly hatched first instar larvae of A. stephensi serve as sources have been established. The various outgrowths which appear at different times during the cultivation period could be distinguished morphologically as large and small epithelial cells, fibroblast-like cells, neuron-like cells, polygonal cells, two types of myoblast-like cells and noncellular and cellular vesicles. Viability of the various outgrowths for a period of approximately 60 days without change of nutrient medium has been observed. Primary cultures in nutrient medium adjusted at various pH (6.5, 7, 8, and 8.5) and incubated either at a temperature of 21°C or 27°C exhibit a similar pattern of outgrowth and degree of viability (Appendix 1:32).

Sporogonic Cultivation

It was observed in our laboratory that in blood sterilly withdrawn from a P. berghei infected rodent and stored for 24 hours at room temperature, exflaggelation of the microgametocytes and fertilization of the macrogametocytes occurred giving rise to ookinete formation in the absence of any contact with mosquitoes (Appendix 1:2). Attempts to exploit this finding with the aim of cultivating sporogonic forms of P. berghei have continued intermittently since the initial observation.

More recently, progress in this area has been substantially facilitated by (1) the use of a technique to induce high gametocytemias with an immunodepressant, and (2) the availability of several modified insect tissue culture media and a variety of long-term primary cell cultures. These media

and cultures are being screened for their ability to support ookinetes in a viable condition and to encourage oocyst development *in vitro* (Appendix 1:32).

Trophozoite Cultivation

Several groups during the past 30 years have reported on attempts to cultivate the red blood cell stages of mammalian malaria, but achievements in this area have been modest. At best, two schizogonic stages have been observed to occur in "cultures" consisting primarily of infected blood maintained in a physiological solution. The approach being adopted by this group is different and is based upon the evidence which indicates that in nature, reinfection of normocytes takes place largely in the blood sinusoids rather than in the general circulating system. It appears likely that parasites are "ingested" by or may penetrate prenormocytes preferentially in restricted areas of the spleen or bone marrow where erythropoesis takes place. Other evidence, including ultrastructural studies, suggest that the plasmodial parasite may well flourish in cell types other than erythrocytes.

On the basis of the above assumptions a screen of various mammalian cells (which have already been adapted to *in vitro* culture procedures) are being tested for their ability to host the trophozoite stages of malaria. The screen also includes cell fusion techniques. Cell fusion, which may occur spontaneously between a small percentage of different cell types mixed in culture, can be greatly enhanced by the use of certain viruses or chemicals. This approach has already yielded some data indicating that the fusion and proliferation of merozoites occurs in non-erythrocytes.

Future work in this area must involve further exploitation of these preliminary findings, as well as the more traditional approach of malaria

infected red cell maintenance. The future practicality of a malaria vaccine depends in large measure on major technological breakthroughs in the development of *in vitro* cultivation procedures for trophozoite and sporozoite antigens.

OTHER ACCOMPLISHMENTS

In addition to the Specific Performance Objectives outlined above a number of new contributions to the field of malariology have been made. Most of these are summarized in detail in the more than 35 publications which are listed in Appendix 1. A few of the highlights are briefly reviewed below.

Ultrastructural studies

The electron microscope has been a valuable tool during the past five years that it has been available to the project. It has been used to evaluate the various methods of preparation of trophozoite antigen (Appendix 1:24) and sporozoite antigen (Appendix 1:22). These studies on the ultrastructural state of the various plasmodial stages during various treatment processes provide information not otherwise available.

Among other "spin off" resulting from electron microscopic studies has been the discovery of what may be a new stage in the life cycle (Appendix 1:26). The detection of this form may provide further insights into the differentiation and metamorphosis of malaria.

Ultrastructural studies on the effect of a blood meal on mosquitoes fed on rabbits previously immunized with preparations of mosquito stomach antigens (see page 14 for details) revealed certain striking morphological changes in the mosquito stomach epithelium. Since these observations were made on mosquitoes from a single experiment, the results will have to be

substantiated by repetition of the original experiment. Analysis and comparison of the electron micrographs obtained from mosquitoes fed on a normal rabbit and one that had been immunized with mosquito stomach preparations revealed a structural alteration of the endoplasmic reticulum in the epithelial cells of mosquitoes fed on the immunized rabbit. The usual arrangement of the endoplasmic reticulum in the mosquito stomach epithelium is in the form of numerous complex whorls which open up shortly after a blood meal. Approximately 50 hours post feeding the whorls reform. This sequence of events appears to have taken place in mosquitoes fed on the control rabbit. The endoplasmic reticulum which is involved in the synthesis of digestive enzymes, failed to reorganize in the cells of four of the five mosquitoes examined which had been fed on the immunized animal. The inability of the endoplasmic reticulum to resume its pre-blood meal state has many implications concerning the ability of an affected mosquito to digest the blood clot. Those results suggest that anti-mosquito antibody may act on this subcellular organelle and its effects may account for the increased mortality observed in mosquitoes fed on immunized rabbits as compared to those fed on normal rabbits.

The electron microscope has proved to be a crucial device for monitoring and interpreting cellular and tissue development _in vitro._ Ultrastructural analysis of various cells and tissues cultured have aided in the selection of suitable cell types for further experimentation and cultivation for antigen production.

Another area of importance is the use of the electron microscope in assessing pathology of renal and other tissues obtained from immunized and nonimmune animals following challenge infection.

Immunobiologic Studies

The nature of the immune response to malaria has puzzled research workers. As a result of the successful demonstration by British workers in West Africa in the early 1960's that passive transfer of concentrated gammaglobulin from adults to children caused a sharp, but transient, drop in parasitemia, it was felt that the primary immune response was due to the production of humoral antibody. However, this left unexplained numerous facts including the failure to obtain correlation between serologic titers and resistance or susceptibility.

The matter has become further clarified (or complicated!) by the demonstration in our (Appendix 1:37) and other laboratories that the mechanism of resistance to the blood stages of P. berghei involved a large component of "cell mediated immunity". On the other hand, similar work in our laboratory with immunity to sporozoite stages indicate that humoral rather than cell mediated immunity is involved in resistance to the sporozoite stages. The work briefly referred to above involved the use of antithymocyte serum and also cell transfer experiments. These findings underline the complex nature of the mechanism of resistance to malaria and indicate why it is so difficult to apply information uncritically from other fields of infectious immunology or from what is known about the host-parasite relations of one stage of malaria to another stage.

Attenuated Malaria Strains

During the course of the cyclic transmission of rodent malaria, samples of the infected blood stages were stored in liquid nitrogen as stabilate material for comparative studies. These studies revealed

considerable variations of virulence between the various lines. An in-depth analysis has confirmed the phenomenon and suggested the possibility of selecting stable lines of malaria which might be used as a live vaccine (Appendix 1: 5, 7, 8). The genetics of the change from relative virulence to avirulence remains to be elucidated but the potential application is intriguing. Avirulent lines of P. berghei which result in as high as 80% survival were found to protect mice against virulent lines which otherwise regularly produce virtually 100% mortality. Of additional interest in this regard are the observations on cross-protectivity between two species of rodent malaria, P. berghei and P. chabaudi . It may well be that a combination of an avirulent heterologous species of malaria can be used to protect against a virulent species.

Antimalaria Antibiotic

In connection with the comparative studies on virulent and avirulent malaria lines, the aniviral antibiotic, rifampicin, was administered to determine whether the possible presence of a virus might have contributed to the pathogenicity of the plasmodial infection in mice. As a result, prolongation of patency and increased survival rates were observed in the antibiotic-treated mice. Subsequent follow-up studies confirmed that rifampicin has a sparing effect on malaria infection (Appendix 1:6). This was the first report of such activity.

SUMMARY AND OUTLOOK

The work reviewed in this final report gives substantial support to the concept that vaccination against malaria is a feasible reality. Numerous difficult practical problems require to be solved, but these are largely of

a technological nature rather than of problems requiring the need to establish new principles of science.

High amongst the technological "breakthroughs" which must take place before a practical malaria vaccine can be achieved is the development of adequate in vitro cultivation procedures for the production of sporozoite and trophozoite antigens. While some significant advances have been made in this area, in our own laboratories and by other workers, it remains a field in which only a small handful of research personnel are working throughout the world. Indeed, it is remarkable that the degree of progress so far reported has been achieved considering the small size of the effort.

Another major task which remains is the isolation and characterization of the active immunogenic components of both the sporozoite and trophozoite antigens. We may derive encouragement from the fact that relatively crude antigenic preparations have proven to be effective vaccines and can confidently expect greater immunizing effectiveness as purification proceeds. We should be aware, however, that the high probability that these antigens are membrane components raises the need to engage the services of sophisticated researchers familiar with recent developments in the dynamic field of biological membranes. The molecular structure, biophysics and biochemistry of biological membranes is only now beginning to be revealed. It is very probable that future purification of malarial antigens will depend upon, and contribute to, progress in this related field.

The means of achieving an effective immunity to malaria is complicated by several factors. The need for a polyvalent antigen vaccine is apparent from data provided by both the mouse and primate model systems. In those

animals which were successfully protected with trophozoite antigen, many suffered substantial blood loss even though very low parasitemias were apparent. During this period of stress, the malaria-protected animal might succumb to a secondary invader, as actually occurred in one monkey of the primate trials reported here (Appendix 2: Fig. 4). Clearly, the primary need is to prevent infection by vaccination with sporozoite antigen, although the immunity to this stage alone would not inhibit progress of the disease if an infection broke through the anti-sporozoite barrier. A combination of both antigens to stimulate resistance to the initiation of the infection and to suppress an infection after it has reached the blood stage is obviously highly desirable. However, immunization with multiple antigens injected at the same time is known to be less effective than presenting each antigen by itself. The effectiveness of a polyvalent vaccine can be markedly improved by purifying the active antigens so that it is free of nonfunctional antigenic contaminants. Purification of the malarial antigens, therefore, is an important step to be accomplished.

Another factor affecting the means of achieving an effective immunity to malaria with a polyvalent vaccine is the evidence that indicates that the type of immune mechanism acting against sporozoites is different from that involved against trophozoites. Data produced in our laboratory indicate that while cell-mediated mechanisms are an important component of anti-trophozoite immunity, they appear to play very slight or no role in anti-sporozoite immunity. Whether this distinction will be confirmed in human malaria remains to be ascertained but the demonstrated need for Freund's complete adjuvant in the primate model system tends to support the conclusion that cell-mediated immunity is an important consideration for successful trophozoite vaccination. An acceptable equivalent adjuvant capable of

stimulating the type of cellular response which Freund's adjuvant is known to produce is urgently needed. How such an adjuvant would affect the immunizing qualities of a sporozoite antigen, if it were incorporated into the same adjuvant with the trophozoite material, is unclear. It is known, however, that optimal immunizing conditions differ for the stimulation of humoral and cell-mediated immunity.

The problems listed in this summary which require solution before an effective human malaria vaccine can be developed are formidable. Indeed, the array of obstacles to be overcome can be discouraging to even the most optimistic among us. However, it is instructive to look back only a decade or so and to observe the impressive progress which has been made in understanding malarial immunity and in the development of model vaccine systems. Also, a close examination of the problems reveal that they are not substantially more complex or demanding than comparable ones which required resolution before the spectrum of viral vaccines now in general use become a reality. The development of tissue culture systems, antigen harvesting and purification, and methods of storage and effective immunization were and are being regularly established by virologists and immunologists. This retrospective view gives confidence that a human malaria vaccine can be produced when adequate resource is applied to this goal.

PUBLICATIONS

1. Alger, N. E. (1968). Individually humidified closed plastic cages for rearing anopheline mosquitoes. Mosquito News, 28(1): 121,122.

2. Alger, N. E. (1968). In vitro development of Plasmodium berghei ookinetes. Nature. 218(5143): 774.

3. Alger, N. E. and Undeed, A. H. (1970). The control of a microsporidan, Nosema Sp., in an anopheline colony by an egg-rinsing technique. Journal of Invertebrate Pathology, 15: 321-327.

4. Alger, N. E. and Cabrera, E. J. (1971). An increase in death rate of Anopheles stephensi fed on rabbits immunized with mosquito antigen. Journal of Economic Entomology. 65: 165-168.

5. Alger, N. E. Branton, M. and Silverman, P. H. (1971). Plasmodium berghei NK65 in the inbred A/J mouse: Variations in virulence of P. berghei demes. Journal of Protozoology.18: 598-601.

6. Alger, N. E., Spira, D. T. and Silverman, P. H. (1971). Inhibition of rodent malaria in mice by rifampicin. Nature. 227(5256): 381-382.

7. Alger, N. E., Branton, M. and Silverman, P. H. (1972). Plasmodium berghei NK65 in the inbred A/J mouse. Immunity in the A/J mouse naturally recovered from NK65C and challenged with NK65E. Journal of Protozoology. (In press).

8. Alger, N. E., Branton, M., Harant, J., Willis, L. and Silverman, P. H. (1972). Plasmodium berghei NK65 in the inbred A/J mouse. Age immunity in the female retired breeder A/J mouse. Journal of Protozoology (In press).

9. Alger, N. E., Harant, J. A., Willis, L. C., Jorgensen, G. M. (1972). Sporozoite and normal mosquito salivary gland induced immunity in malaria. Nature (In press).

10. Cabrera, E. J.(1968).Effect on the death rate of anopheline mosquitoes which fed on immunized rabbits. Master's Degree Thesis.

11. Cabrera, E. J. and Alger, N. E. (1971). Transfer of adoptive immunity to Plasmodium berghei: A comparison of the routes of injection. Journal of Protozoology. 18: 596-597.

12. D'Antonio, L. E., Alger, N. E. and Mathot, C. (1969). Temporal relation between a serum electroadsorbing fraction and the course of acute Plasmodium berghei malaria in rats. American Journal of Tropical Medicine and Hygiene. 18: 866-871.

13. D'Antonio, L. E., Spira, D. T. and Silverman, P. H. (1969). A model system for the study of artificially induced resistance to malaria. Nature. 223-:507-509.

14. D'Antonio, L. E., Fu, R. C., Dagnillo, D. M. and Silverman, P. H. (1969). Induction of resistance to malaria with temperature inactivated plasmodially infected blood. Journal of Protozoology. 16(Suppl). Abstract. 51:17.

15. D'Antonio, L. E., Fu, R. C., Dagnillo, D. M. and Silverman, P. H. (1969). The induction of resistance to malaria with a partially isolated plasmodial fraction. Journal of Protozoology, 16(Suppl) Abstract 52:17.

16. D'Antonio, L. E. and Silverman, P. H. (1970). Malaria resistance induced by plasmodial particulate fraction. Federation Proceedings. 29(2): 2206.

17. D'Antonio, L. E., Spira, D. T., Fu, R. C., Dagnillo, D. M. and Silverman, P. H. (1970). Malaria resistance: Artificial induction with a partially purified plasmodial fraction. Science. 168(3935): 1117-1118.

18. D'Antonio, L. E. and Silverman, P. H. (1971). Technical comment. Malaria resistance. Artificial induction with a partially purified plasmodial fraction. Science. 171: 1176.

19. D'Antonio, L. E., Dagnillo, D. M. and Silverman, P. H. (1971). Vaccination of Rhesus monkeys against Plasmodium knowlesi malaria with a partially purified plasmodial fraction. Federation Proceedings. (Abstract) 30(2) p. 303, No. 617.

20. D'Antonio, L. E. (1972). Plasmodium: A resume of the isolation of a vaccine fraction by the French pressure cell technique. Experimental Parasitology. 31: 75-81.

21. D'Antonio, L. E. (1972). Plasmodium berghei: Vaccination of mice against malaria with heat inactivated parasitized blood. Experimental Parasitology. 31: 82-87.

22. Jakstys, B. P., Alger, N. E., Harant, J. A. and Silverman, P. H. (1972). Ultrastructural analysis of P. berghei sporozoite antigen. Proceedings of the Society of Protozoologists 25th Annual Meeting. (In press).

23. Killby, V. A. A. and Silverman, P. H. (1969). Fine structural observations of the erythrocytic stages of Plasmodium chabaudi Landau, 1965. Journal of Protozoology. 16: 354-370.

24. Killby, V. A. A. and Silverman, P. H. (1969). Isolated erythrocytic forms of Plasmodium berghei: an electronmicroscopical study. American Journal of Tropical Medicine and Hygiene. 18(6): 836-859.

25. Killby, V. A. A. and Silverman, P. H. (1971). Preliminary studies on the use of ferritin-conjugated antibodies to Plasmodium berghei. Journal of Protozoology. 18: 73-79.

26. Killby, V. A. A. and Silverman, P. H. (1971). Ultrastructure of an atypical erythrocytic form of Plasmodium berghei. Journal of Protozoology. 18: 51-57.

27. Rosales-Ronquillo, M. C. and Silverman, P. H. (1972). Laboratory studies on the bionomics of Anopheles stephensi Liston (Diptera, Culicidae). I. Hatch rate of eggs maintained at various low temperatures. (In preparation).

28. Rosales-Ronquillo, M. C. and Silverman, P. H. (1972). Laboratory studies on the bionomics of Anopheles stephensi Liston (Diptera, Culicidae). II. Survival rate of the developmental stages obtained from low temperature treated eggs. (In preparation).

29. Rosales-Ronquillo, M. C. and Silverman, P. H. (1972). Laboratory studies on the bionomics of Anopheles stephensi Liston (Diptera, Culicidae). III. Reproductive capacity and longevity of adults reared from low temperature treated eggs. (In preparation).

30. Rosales-Ronquillo, M. C., Simons, R. W. and Silverman, P. H. (1972). Aseptic rearing of Anopheles stephensi Liston. (In preparation).

31. Rosales-Ronquillo, M. C., Simons, R. W. and Silverman, P. H. (1972). Cell cultures derived from mosquito tissues, Anopheles stephensi Liston. III International Colloquium on Invertebrate Tissue Culture. Bratislava, Czechoslovakia, June 21-25, 1971). (In press.)

32. Rosales-Ronquillo, M. C., Simons, R. W. and Silverman, P. H. (1972). Long-term primary culture of cells of the mosquito Anopheles stephensi Liston. Annals of Entomological Society. 65(3): 721-729.

33. Schenkel, Robert H., Simpson, Gary L. and Silverman, Paul H. (1972). Vaccination of Rhesus monkeys (M. mulatta) against Plasmodium knowlesi by use of nonviable antigen. (In preparation).

34. Silverman, Paul H. (1970). Vaccination: Progress and problems. Chapt. 37 In, Immunity to Parasitic Animals, ed. by J. Singer and G. Jackson, Appleton, Century and Crofts, New York. Vol. 2: pp. 1165-1185.

35. Simons, R. W., Rosales-Ronquillo, M. C. and Silverman, Paul H. (1971). Cell movement in vitro using mosquito tissues of Anopheles stephensi Liston. (Presented at Midwest Regional Developmental Biology Conference, Elmhurst, Ill., April 23-24, 1971) Abstract.

36. Simons, R. W., Rosales-Ronquillo, M. C. and Silverman, P. H. (1972). Long-term maintenance in nutrient medium of adult organs taken from aseptically reared mosquitoes, Anopheles stephensi Liston. III International Colloquium on Invertebrate Tissue Culture. Bratislava, Czechoslovakia, June 21-25, 1971). In press).

37. Spira, D. T., Silverman, P. H. and Gaines, C. (1970). Antithymocyte serum effects of Plasmodium berghei infection in rats. Immunology, 19(5): 579-766.

38. Undeen, A. H. and Alger, N. E. (1971). Density gradient method for fractionating microsporidan spores. Journal of Invertebrate Pathology. 18(3): 419-420.

39. Vavra, Jiri and Undeen, A. H. (1970). Nosema algerae n.sp. (Cnidospora, microsporida) a pathogen in a laboratory colony of Anopheles stephensi Liston (Diptera, Culicidae). Journal of Protozoology. 17(2): 240-249.

LEGENDS

Figure 1. Flow diagram illustrating the procedure for preparation of partially purified trophozoite antigen from *Plasmodium berghei* and *P. knowlesi*. The French pressure cell (FPC) is operated at controlled pressures by means of an automatic hydraulic press.

Figure 2. The average parasitemias of immunized and nonimmune DDS and A/J mice following challenge inoculation with viable *Plasmodium berghei*.

Figure 3. The average red blood cell response of immunized and nonimmune A/J mice following challenge inoculation with viable *Plasmodium berghei*.

Figure 4. Red blood cell response of immunized and nonimmune Rhesus monkeys following challenge inoculation with viable *Plasmodium knowlesi*. Both nonimmune monkeys (1223 and 4168) died (D).

Figure 5. Parasitemias of immunized and nonimmune Rhesus monkeys following challenge inoculation with viable *Plasmodium knowlesi*.

Figure 6. Summary of mice surviving challenge inoculation with 2,000 viable sporozoites following immunization with frozen and thawed inactivated sporozoites of *Plasmodium berghei* or with salivary gland material from uninfected *Anopheles stephensi* mosquitoes.

Figure 7. Summary of mice surviving challenge inoculation with 2,000 viable sporozoites following immunization with heat inactivated sporozoites of *Plasmodium berghei* or with salivary gland material from uninfected *Anopheles stephensi* mosquitoes.

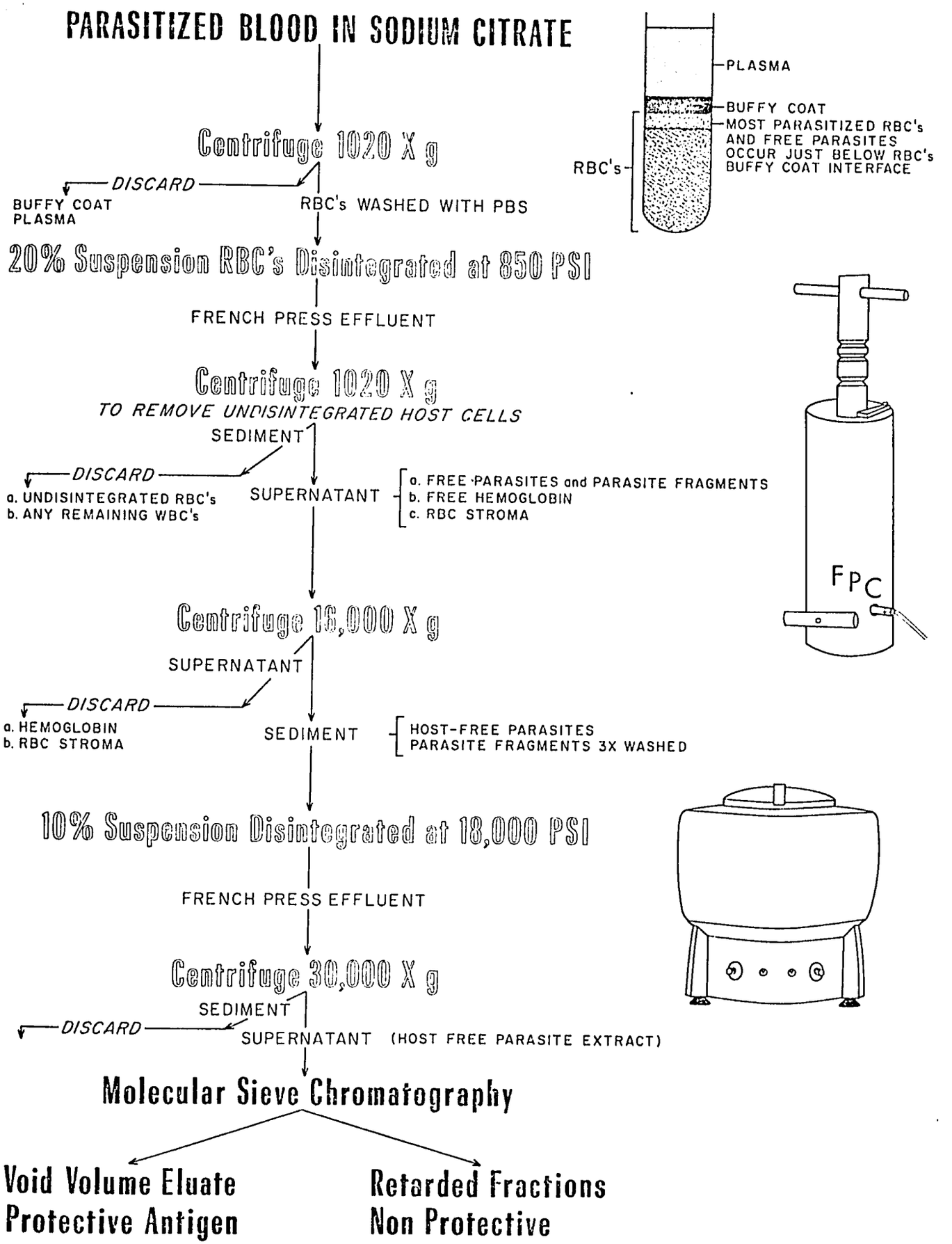
PARASITIZED BLOOD IN SODIUM CITRATE
PLASMA
BUFFY COAT
MOST PARASITIZED RBC's AND FREE PARASITES OCCUR JUST BELOW RBC's BUFFY COAT INTERFACE
RBC's
Centrifuge 1020 X g
DISCARD
BUFFY COAT
PLASMA
RBC's WASHED WITH PBS
20% Suspension RBC's Disintegrated at 850 PSI
FRENCH PRESS EFFLUENT
Centrifuge 1020 X g
TO REMOVE UNDISINTEGRATED HOST CELLS
SEDIMENT
DISCARD
a. UNDISINTEGRATED RBC's
b. ANY REMAINING WBC's
SUPERNATANT
a. FREE PARASITES and PARASITE FRAGMENTS
b. FREE HEMOGLOBIN
c. RBC STROMA
FPC
Centrifuge 16,000 X g
SUPERNATANT
DISCARD
a. HEMOGLOBIN
b. RBC STROMA
SEDIMENT
HOST-FREE PARASITES
PARASITE FRAGMENTS 3X WASHED
10% Suspension Disintegrated at 18,000 PSI
FRENCH PRESS EFFLUENT
Centrifuge 30,000 X g
SEDIMENT
DISCARD
SUPERNATANT (HOST FREE PARASITE EXTRACT)
Molecular Sieve Chromatography
Void Volume Eluate
Protective Antigen
Retarded Fractions
Non Protective

FIG. 1

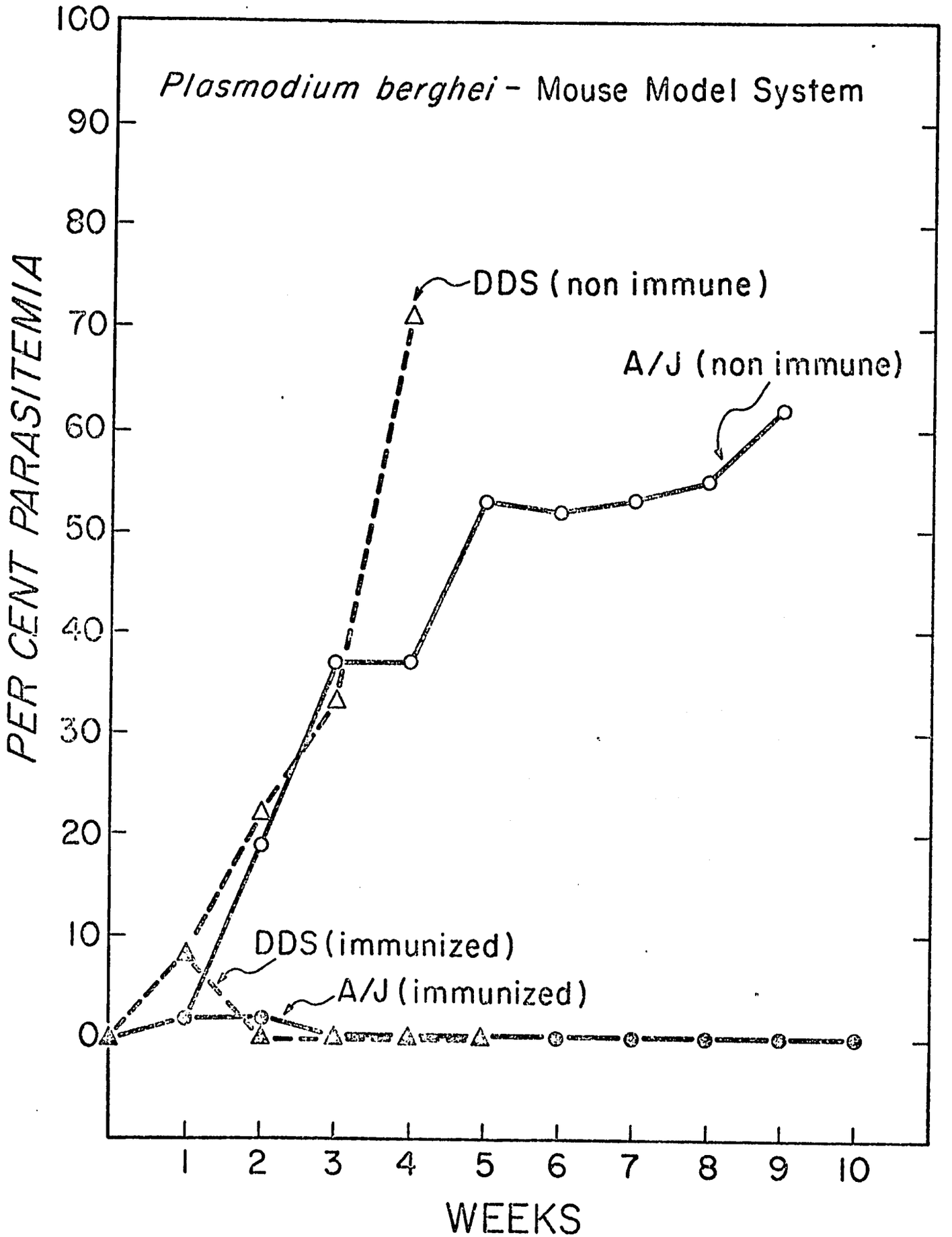
Plasmodium berghei - Mouse Model System
PER CENT PARASITEMIA
100
90
80
70
60
50
40
30
20
10
0
DDS (non immune)
A/J (non immune)
DDS (immunized)
A/J (immunized)
1
2
3
4
5
6
7
8
9
10
WEEKS

FIG. 2

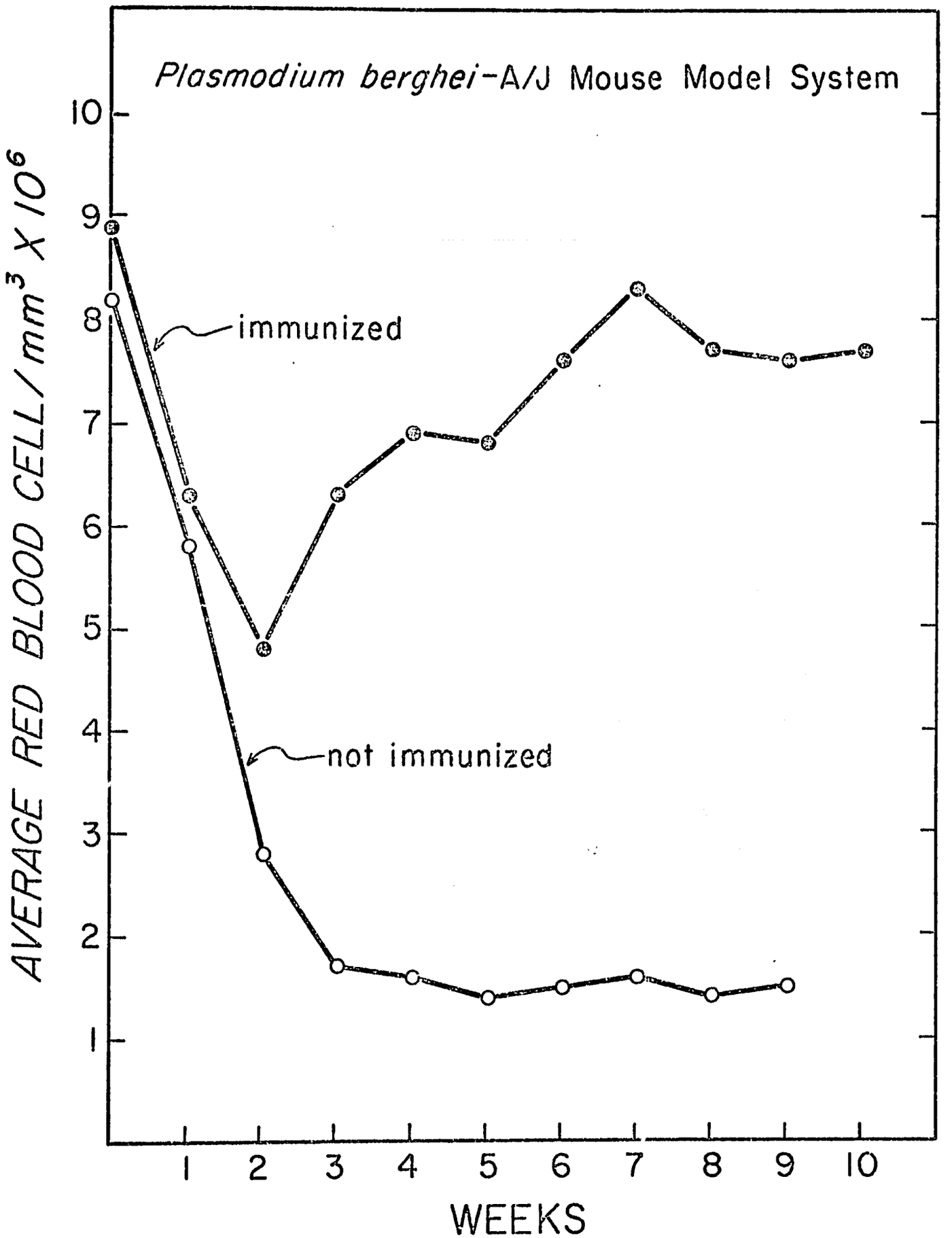
Plasmodium berghei-A/J Mouse Model System
AVERAGE RED BLOOD CELL/mm³ X 10⁶
10
9
8
7
6
5
4
3
2
1
immunized
not immunized
1
2
3
4
5
6
7
8
9
10
WEEKS

FIG. 3

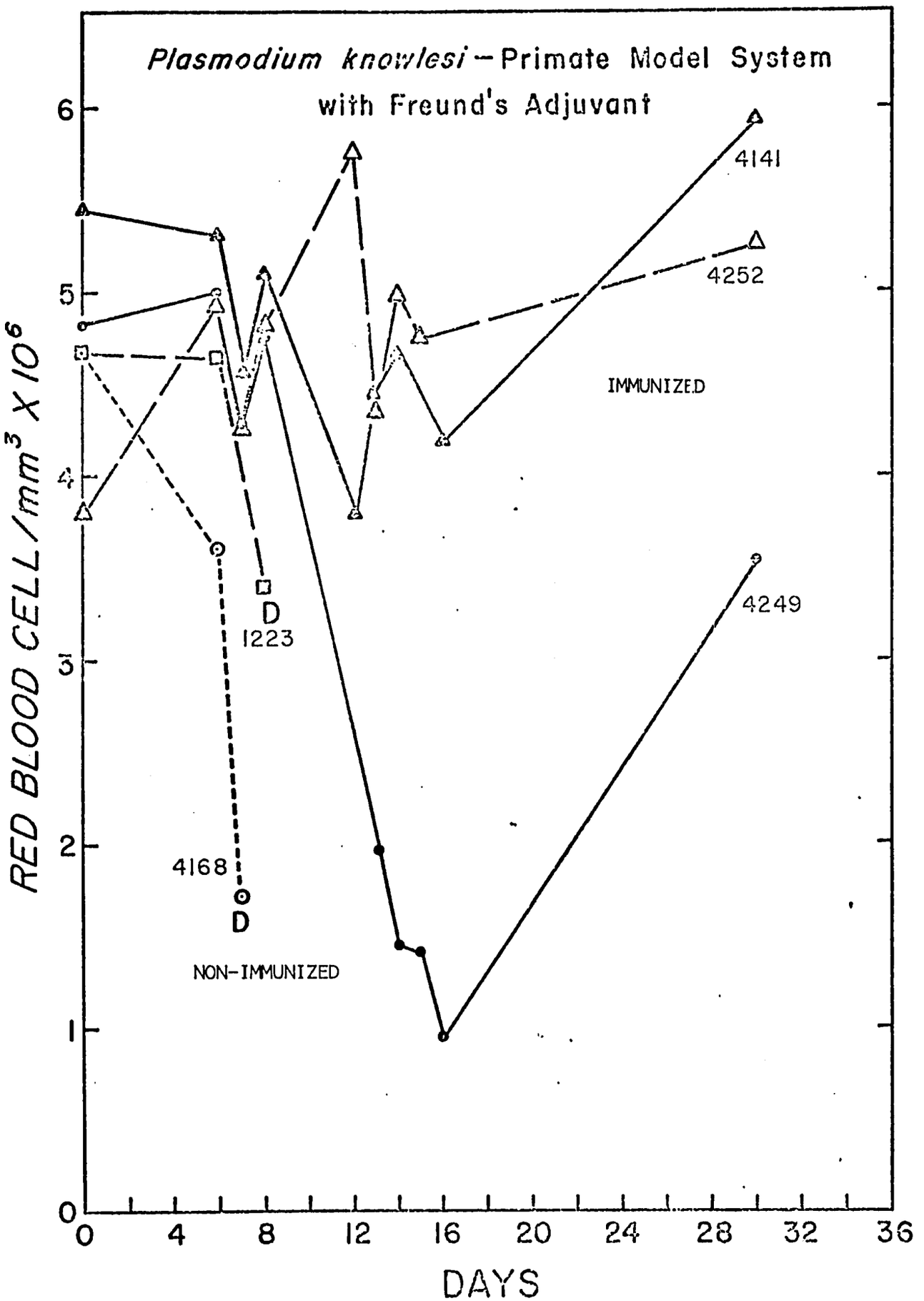
Plasmodium knowlesi – Primate Model System
with Freund's Adjuvant
RED BLOOD CELL/mm³ X 10⁶
DAYS
0
1
2
3
4
5
6
0
4
8
12
16
20
24
28
32
36
4141
4252
IMMUNIZED
4249
D
1223
4168
D
NON-IMMUNIZED

FIG. 4

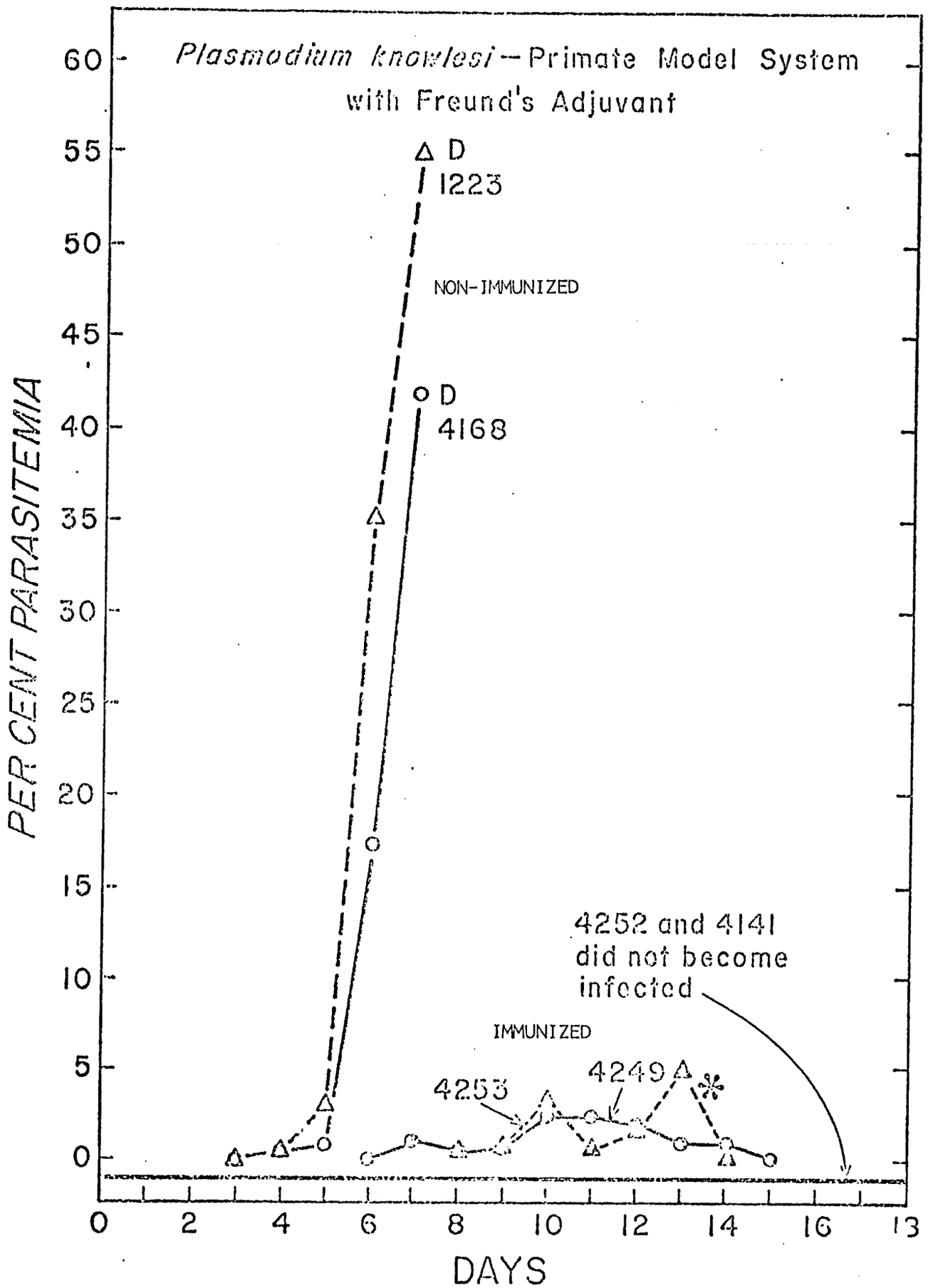
Plasmodium knowlesi—Primate Model System
with Freund's Adjuvant
PER CENT PARASITEMIA
DAYS
D
1223
NON-IMMUNIZED
D
4168
4252 and 4141
did not become
infected
IMMUNIZED
4253
4249
0
5
10
15
20
25
30
35
40
45
50
55
60
0
2
4
6
8
10
12
14
16
13

FIG. 5

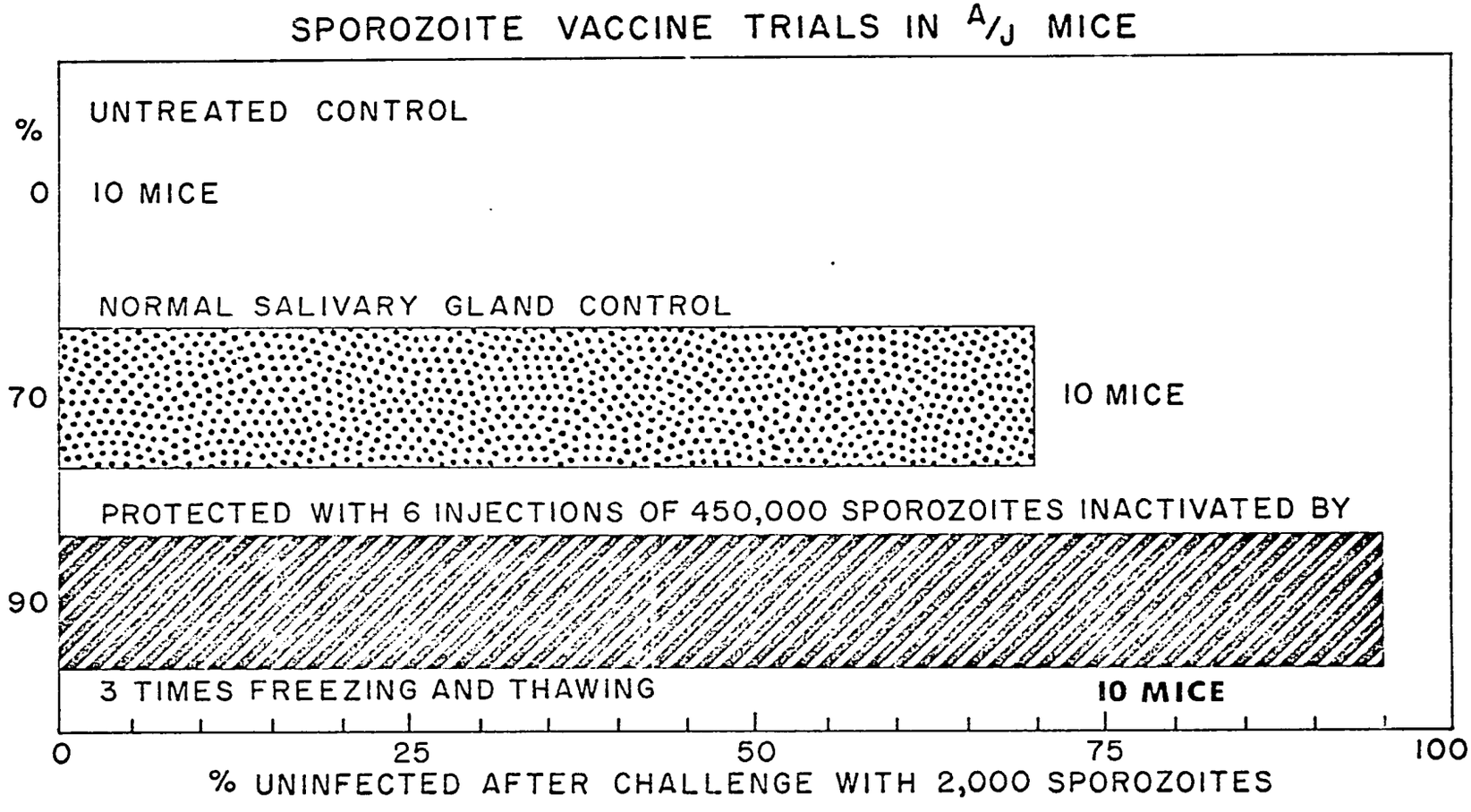
SPOROZOITE VACCINE TRIALS IN A/J MICE
%
0
UNTREATED CONTROL
10 MICE
NORMAL SALIVARY GLAND CONTROL
70
10 MICE
PROTECTED WITH 6 INJECTIONS OF 450,000 SPOROZOITES INACTIVATED BY
90
3 TIMES FREEZING AND THAWING
10 MICE
0
25
50
75
100
% UNINFECTED AFTER CHALLENGE WITH 2,000 SPOROZOITES

FIG. 5

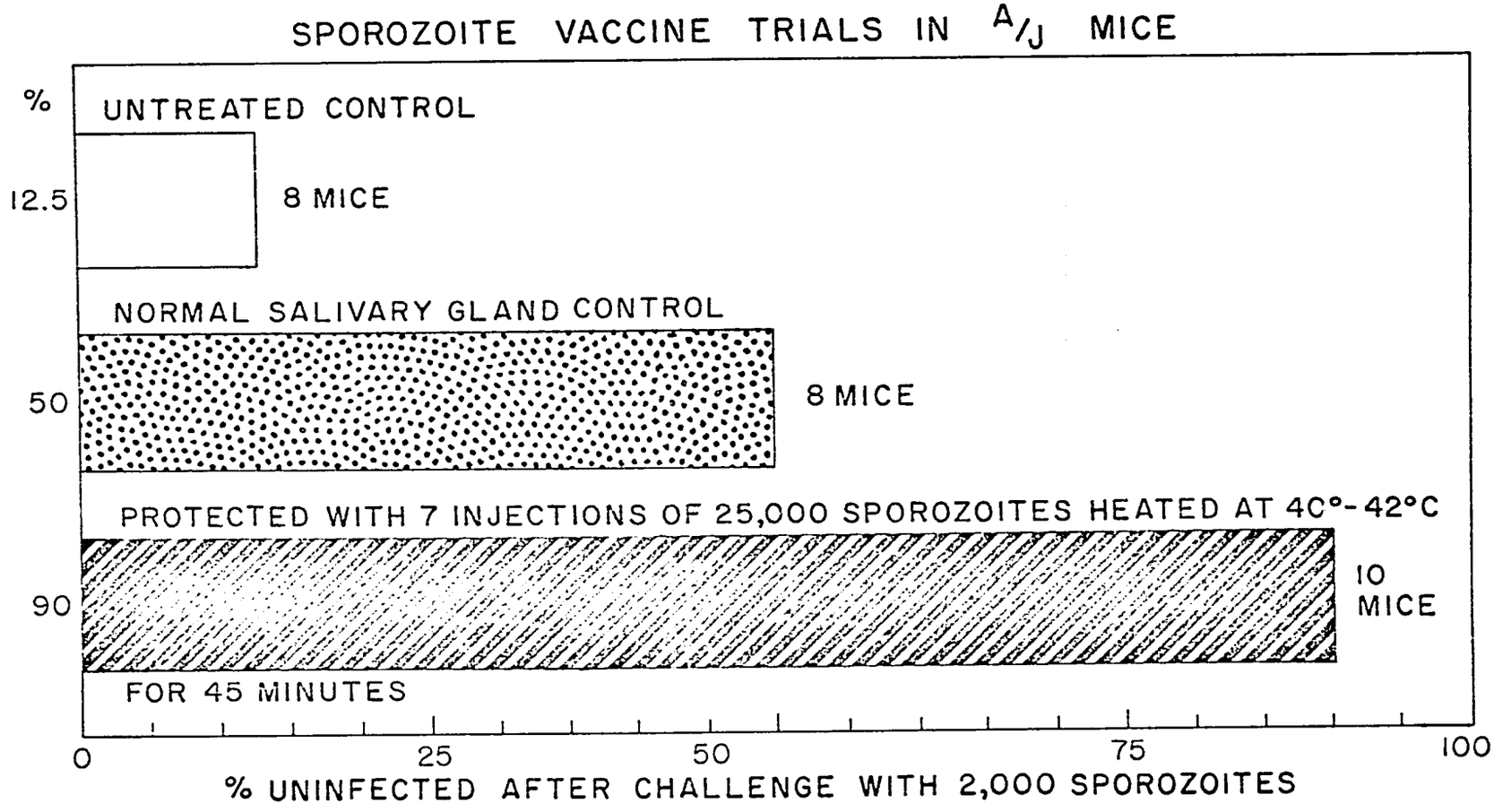
SPOROZOITE VACCINE TRIALS IN A/J MICE
%
UNTREATED CONTROL
12.5
8 MICE
NORMAL SALIVARY GLAND CONTROL
50
8 MICE
PROTECTED WITH 7 INJECTIONS OF 25,000 SPOROZOITES HEATED AT 40°-42°C
90
10 MICE
FOR 45 MINUTES
0
25
50
75
100
% UNINFECTED AFTER CHALLENGE WITH 2,000 SPOROZOITES

FIG. 7