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SUPERINFECTION AND PROTECTIVE EXPERIMENTS WITH *PLASMODIUM BRASILIANUM* IN MONKEYS

SUPERINFECTION AND PROTECTIVE EXPERIMENTS WITH *PLASMODIUM BRASILIANUM* IN MONKEYS.*

By

WILLIAM H. TALIAFERRO and LUCY GRAVES TALIAFERRO

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Preliminary data on these experiments have already been published by one of us (W. H. Taliaferro, 1932). They form part of a series of investigations on *Plasmodium brasilianum*, a typically quartan parasite, in various species of monkey. Thus, we (1934) have shown that the segmenters ordinarily exhibit 8 to 12, with a range of 4 to 16 nuclei (average 8.5–10); a 3-day periodicity of reproduction is maintained in the spiders, howlers and white throated monkeys throughout the entire course of infection when parasites are present in sufficient numbers to study, with sporulation occurring between 8 a.m. and 4 p.m. except that (1) the first sporulation † is occasionally prolonged to 4 days due primarily to the manipulations of transfer (in 3 of 67 monkeys) and (2) sporulation is frequently prolonged to 4 or 5 days at the time of the crisis (number drop) due to the immune response of the host. Furthermore, the parasite exhibits a course of infection characterized by a more or less acute rise in numbers which sometimes reaches a peak of 300 or more per 10,000 red cells and at other times does not exceed 25 per 10,000 red cells, a sharp or gradual decrease in numbers (crisis), a low-grade infection, and thereafter short periods during which no parasites can be found are frequently interspersed with relapses of varying degrees of intensity. From the first introduction of parasites, a tremendous mortality of parasites occurs at each sporulation (the numbers generally increased 1 to 3 times instead of 8.5 to 10 times) as well as during each intersporulation period, but is

* From the Department of Hygiene and Bacteriology of the University of Chicago and the Gorgas Memorial Laboratory, R. de Panama. This work has been aided by a grant from the International Health Division of the Rockefeller Foundation. The authors wish to thank Dr. H. C. Clark and his staff (in particular Mr. J. Benavides), Dr. P. W. Wilson, Lt. Com., M.C., U.S.N., Mr. Charles Martin, Chief Pharmacist's Mate, U.S.N., and Mr. L. A. Stauber of the University of Chicago for invaluable assistance.

† Parasites were often injected intravenously so that they appeared in the blood immediately.

greatly accentuated at the time of the crisis and thereafter. This greater death rate of the parasites at the time of the crisis which may or may not be associated with the concomitant presence of peculiarly staining and apparently degenerating segmenters is the first evidence of an acquired resistance during which strong plasmodicidal factors are operating. These plasmodicidal factors apparently continue to operate, as the data in this paper show. In infected monkeys, sharp peaks in temperature are often superimposed upon the normal diurnal temperature rhythm provided parasites are sufficiently numerous. For other conclusions and for a review of literature, the original paper may be consulted.

Contributions to the problem of superinfection in malarial infections have been rapidly increasing recently, especially in human and avian infections. With regard to simian malaria, a few sporadic papers have appeared beginning with the work of Flu (1908) and culminating in the extremely illuminating paper by Mulligan and Sinton (1933). All of this literature has been ably reviewed by Mulligan and Sinton (1933) to whose paper the reader is referred. These latter authors, working with 5 strains of *P. knowlesi* and one of *P. inui* var. *cynomolgi*, found an effective immunity to superinfection with homologous strains of the same species, a slight immunity to superinfection between different strains of the same species and no cross immunity to superinfection with *P. inui* var. *cynomolgi* in monkeys infected with *P. knowlesi*.

Materials and methods.

Five species of monkeys are represented in this series of experiments and belong to the following species: *Ateles geoffroyi*, Kuhl 1820, the red spider; *Ateles dariensis*, Goldman 1915, the black spider; *Cebus capucinus*, Linn. 1758, the white throated monkey, *Alouatta palliata inconsonans*, Goldman 1913, the black howler and *Alouatta palliata palliatta*,* the brown howler. They will be designated as RS, BS, W, BlH and H, respectively, and the numbers used are identical with those in previous publications by Clark (1930 and 1931) and us (1932 and 1934).

Infections of *P. brasilianum* from seven naturally infected monkeys were used in this work. For convenience these are considered as different strains.

The same techniques of obtaining parasites and making intravenous injections, of making blood films and staining, of ascertaining numbers

* So classified by Clark and Dunn (1933), but a new species according to Prof. Thomas Barbour of Harvard University.

per 10,000 red cells, of examining the monkeys, etc., were used in this study as were used previously (1934). Similarly, the same method of measuring periodicity was used and briefly consisted in tabulating the occurrence of rings, uninucleated schizonts less than half the size of the blood cell, uninucleated schizonts half or more than half the size of the blood cell and segmenters according to their number of nuclei in a sample of 50 parasites from thin or thick films at regular intervals. Experience showed that the cycle could be reconstructed from blood films made daily at 8 a.m., but that films at 8 a.m. and 8 p.m. were preferable. These data are summarily treated in the accompanying graphs as the percentage of parasites with 5 or more nuclei occurring at each observation-period. Such a procedure shows the periodicity of the cycle fairly accurately since parasites with 5 or more nuclei occur during approximately 12 hours in the 72 hour-reproductive period. It should always be borne in mind, however, that the peak in the occurrence of forms with 5 or more nuclei antedates by about 4 hours, as ascertained by previous work, the actual peak in sporulation, i.e., the simultaneous occurrence of multinucleated and merozoite forms. To simplify terminology, "segmenter" will be used to indicate all parasites with 5 or more nuclei.

Experimental work.

The procedure in studying superinfected monkeys has been the same throughout. After a monkey had been found naturally infected or had been experimentally infected and its infection studied, it was reinoculated intravenously with a large number of parasites at a time when there were few or no parasites in its blood. Then, within a few minutes, a blood film was made from which the number of parasites injected could be ascertained, and thereafter, blood films were made at frequent intervals for a month and weekly thereafter until the animal was killed. This is exemplified in one of the preliminary experiments which is shown graphically in figure 1.

W46, an infant, entered the laboratory on 8/5/30 and after 66 negative blood film examinations was injected intravenously with parasites on 4/10/31 so that immediately afterwards there were 3 parasites per 10,000 red cells in its blood. These gradually increased for 11 days, except for the expected periodic decreases during intersporulation periods, as previously described, until on 4/21 at 8 p.m. there were 23 parasites per 10,000 red cells. Thereafter, they rapidly decreased. Throughout the increase in numbers (4/10–4/21) and during the initiation of the crisis (4/23) sporulation was regular, but as the crisis proceeded (4/26) sporulation was irregular in that instead of occurring

every 3rd day, it consumed the greater portion of 2 days. (In figure 1, see continuous presence of segmenters on 4/26 and 4/27 as indicative of sporulation.) When there were less than 1 per 10,000 red cells, additional parasites were introduced intravenously from W21 on 4/30 at 2 p.m. so that there were 31 per 10,000 red cells in the blood of this monkey immediately afterwards (10 times the number originally in-

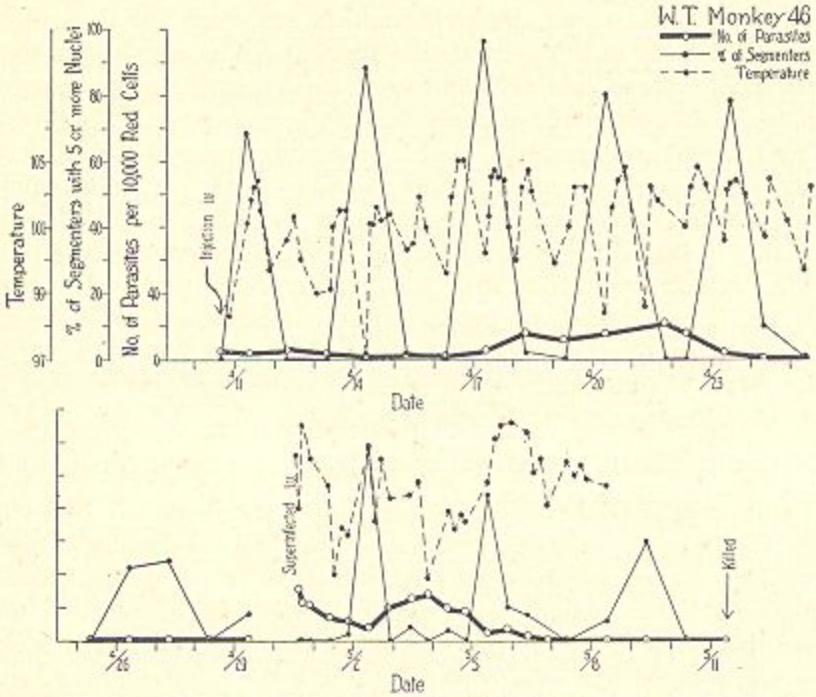

FIG. 1. The number of parasites per 10,000 red cells and percentage of segmenters after initial infection and after superinfection of *P. brasilianum* in white throated monkey 46. (The temperature curve is also given for most of this time.) Note that in spite of the fact that ten times as many parasites were injected intravenously for the superinfection as for the initial infection, the crisis occurred more precipitously as indicated by the drop in numbers and irregularity in sporulation in the superinfection (5/5) than in the initial infection (4/23–4/27) and the numbers decreased more rapidly.

jected). Yet in 7 days these had decreased to 1 per 10,000 red cells (they increased from 9 to 28 as the result of sporulation on 5/2) and continued to decrease until on 5/11 when the animal was killed none was found in thick film. For 4 days after superinfection, sporulation was approximately regular, but on 5/5, occurred continuously for 24 hours and then instead of occurring at 8 a.m. on 5/8 (the next 3-day

interval), it occurred after 8 p.m. that night and throughout the major portion of the following day. On 5/11 when parasites could no longer be found in the blood, the animal was killed for a study of its tissues. In accordance with previous work, it may be noted that the diurnal temperature rhythm, encountered in normal monkeys, was not materially altered in this infected monkey until the time of the crisis during superinfection (5/5).

This sequence of events is interesting from several standpoints. In the first place, this white throated monkey showed the lowest grade initial acute attack of all the white throated monkeys studied, and yet, upon superinfection, the parasites were summarily disposed of. In the second place, even though the monkey was superinfected only 20 days after it had been subjected to an initial infection, and even while parasites were still present, the parasites completely disappeared in 11 days. The objection that it had been previously infected can be strongly refuted, though not absolutely proved, by the fact that it was an infant and showed 66 negative and no positive examinations prior to infection. In the third place, a typical crisis, as indicated by a drop in numbers and an irregularity in sporulation, was manifested sooner after superinfection (5th day) than after initial infection (16th day).

The superinfection of a monkey which had undergone a more acute and longer initial infection may be exemplified by BS91. This monkey was injected intraperitoneally on 3/19/31 after 14 negative blood examinations with so few parasites from naturally infected RS3 and 16 that they did not appear in its blood until 21 days after infection and not in appreciable numbers until 29 days after infection. From this time on (4/17) until 47 days after infection (5/5) detailed data were collected (fig. 2) and show that the typical acute rise in numbers reached a peak of 155 per 10,000 red cells on 4/23 at 8 p.m. and then gradually decreased for 17 days until they could no longer be found in thick or thin blood films. Throughout this entire rise and fall in numbers, sporulation occurred with fine precision although atypical segmenters were numerous from 4/27 to 4/30 (crisis). The temperature curve in the monkey during this time illustrates the stiletto-like peaks which interrupted the customary diurnal rhythm at each sporulation period (4/17, 20, 23, 26) when parasites were numerous and the absence of peaks when numbers were fewer (4/29). At 4 p.m. on 6/1 after a latent period of about 2 weeks, this monkey was superinfected intravenously from W21 and 141 (see controls in table 1) with 15 cc. of heavily parasitized blood cells in 7 cc. of saline so that immediately afterwards it showed 30 intermediate schizonts per 10,000 red cells

in its blood. In 20 hours these had decreased to 9, in 44 hours to 6, and after the first sporulation (68 hours) had further decreased to 1 showing that sporulation was ineffectual in increasing the numbers. Thereafter parasites persisted for 37 days, but never exceeded 2 per 10,000 red cells. Throughout this period sporulation was maintained

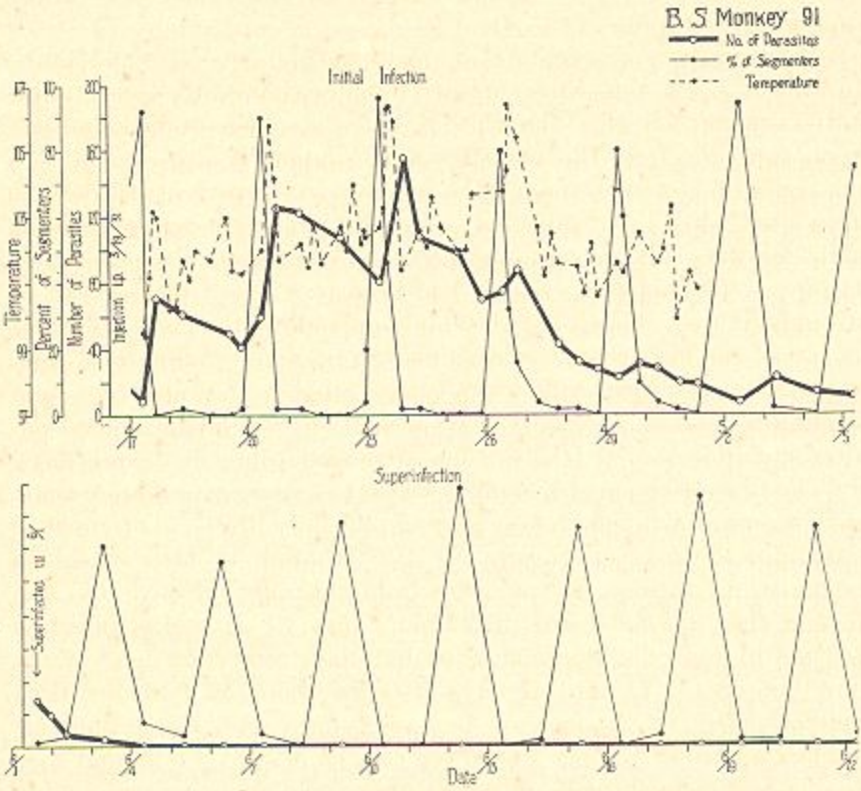

FIG. 2. The number of parasites per 10,000 red cells and percentage of segmenters during a portion of the initial infection and after superinfection of *P. brasilianum* in black spider 91. (The temperature curve is also given for a portion of the initial infection.) Note that a small intraperitoneal dose of parasites was given initially which appeared in the blood eventually and set up a heavy infection whereas a large intravenous dose of parasites was given for the superinfection which quickly decreased and eventually disappeared. Sporulation was maintained regularly throughout.

regularly as ascertained by thick film examinations. The actual dates on which it occurred were regular with respect to the parasites used in the superinfecting dose (6/3, 6/9, etc.) and not with respect to those initially infected (6/1, 6/4, etc., provided sporulation had progressed

uninterruptedly). In other words, the parasites which persisted were probably those of the superinfection-dose and did not represent a relapse of those initially injected. Weekly observations replaced daily ones after 7/7.

Data for one other black and 5 red spiders which were also superinfected occur in table 1. Of these, 5 were naturally infected for more than 74 to 477 days and one had been experimentally infected for 33 days (BS144). The acute attack in the former occurred before their entrance into the laboratory. During the period of observation prior to superinfection, RS94 and 364 had shown none or very few parasites (never more than 20 parasites in a 5-minute thick film survey) in their blood in 54 and 44 examinations, respectively. RS16 had shown none or very few until it was splenectomized on 3/31/31 after which a relapse ensued similar in every respect to the acute infection, just described for S91, which reached a peak of 150 per 10,000 red cells on 4/25 at 8 p.m. and then decreased but never completely disappeared prior to superinfection (thick films showed from 12 to 90 in a 5-minute examination). RS3 and 4 showed alternate periods of latency and slight relapses, but were consistently negative for 23 and 19 examinations extending over 4½ and 1½ months prior to superinfection, respectively. The initial experimental infection in BS144 consistently showed so few parasites and such an aberrant cycle of sporulation that we felt it had been previously naturally infected, an assumption easily warranted since it had only been examined 4 times (all negative) prior to its experimental infection. The most salient parts of these data occur in the first part of table 1 along with the weight, sex and approximate age of the monkey.

All of these animals were superinfected with 15 to 20 cc. of parasitized blood cells so that immediately after the intravenous injection there were from 4 to 30 parasites per 10,000 red cells whereas just before there had been none or extremely few. Just as in the superinfection in W46 and BS91, the majority of the parasites rapidly disappeared. In presenting data on these superinfections in table 1, it was finally decided to give closely spaced number counts during the first sporulation at 6, 20 and 44 hours,* to give only one number count every three days thereafter and to italicize any number count during

* The number of hours before a second sporulation occurred in the superinfected monkeys depended upon the stage of the parasites when superinfected. For example, small rings were used to superinfect S3 at 1 p.m. on 6/1 which sporulated 68–72 hours later, whereas uninucleated schizonts were used to superinfect S94 at 11 a.m. on 5/19 which sporulated 20–24 hours later.

TABLE 1.

Number counts and periodicity† after superinfection with large doses of parasites in 8 monkeys. Similar data after initial infection with similar large doses in 5 monkeys (controls).*

Monkey			Initial infection			Super-infection		After superinfection: No. of parasites per 10,000 red cells												
								Hours				Highest every 3 days								
	Wt. in kg.	Sex and age	Date of peak	No. at peak	Length	Source	Date	1/12	6	20	44									
RS3	3.6	J ♀	NI		477+	W100	6/1	15	13	5	4	1	>1	>1‡	>1	>1	>1	—	OD	
RS4	2.0	J ♀	NI		462+	S95	5/12	4	2	1	1	1	>1‡	>1	—	—	—	—	—	OD
RS16	2.5	J ♀	4/25*	150	407+	S113	6/5	16	13	6	5	5	4	4	1	>1	>1‡	>1	>1	OD
W46	.4	I ♂	4/21	23	20	W21	4/30	31	23	15	9	26	4	*>1*	K					
BS91	3.0	A ♂	4/23	155	111	W141	6/1	30		9	6	1	>1	>1	>1	1	1	1	2	>1
RS94	3.0	A ♀	NI		74+	S95	5/19	6	3	2		*2*	>1	>1	>1‡	>1	>1	OD		
BS144	3.2	A ♂	5/22	14	17	S113	6/5	*9*		*6*	*6*	*4*	>1‡	>1	—	—	OD			
RS364	1.6	J ♂	NI		270+	S395	4/22	12	6	2		4	*>1*	>1‡	>1	—	—	—	—	—
Controls:								After initial infection												
RS95	5.6	A ♀				§	5/1	5		5	4	19	32	*42*	54	76	K			
BS113	5.0	A ♂				S95, 110	5/12	2		2		3	4	11	7	16	31	53	84	43
BS420	2.1	J ♀				S395	3/26	13				95	325	1195	K					
W21	.9	J ♀				S91	4/24	20		15	10	28	104	157	*91*	>1	>1	>1	2	7
W141	.5	I ♂				H40	4/30	2		2		5	~~9~~	16	~~19~~	31	36	~~68~~	~~87~~	~~100~~

A, adult; J, juvenile; I, infant; NI, naturally infected, acute rise not observed; K, killed; OD, daily observations discontinued; —, none in thick film; >1, between .4 per 10,000 red cells and 1 seen in a 5 minute thick film examination.

* All dates 1931 except 1932 for 364 and 420: RS16 was naturally infected; this is the peak in numbers following splenectomy on 3/31

† Occurrence during any 3-day interval of irregularities or delay in sporulation indicated by number count in italics.

‡ Sporulation cycle not determined here and hereafter: numbers too few.

§ Infected with pooled blood from S1, S16, S91, W21, W119.

the sporulation of which an irregularity of sporulation similar to that in W46 occurred.* The number count used was the highest obtained from the afternoon on the day of sporulation—major sporulation if more than one occurred—to the morning of the day of the following sporulation. As may be seen by an examination of this table, RS3 was superinfected with a dose of parasites so that immediately afterwards it showed 15 parasites per 10,000 red cells. These decreased to 13, 5 and 4 in 6, 20 and 44 hours, respectively; during the second sporulation period they never exceeded 1 per 10,000 red cells and thereafter could only be found on thick films. RS4, 94, 144 and 364 behaved similarly. Interestingly enough RS16 which had been splenectomized 64 days prior to superinfection did not get rid of its superinfected parasites as quickly as did the others, but at the end of 5 sporulations showed very few parasites in thick film. The parasites in all of these spiders decreased more rapidly than in W46. This is probably an expression of the fact that their immunity was higher.

The criticism that these infections might show an acute rise in numbers later on when observations were less frequent can be countered by the fact that whenever we gave such an enormous dose of parasites intravenously to monkeys in which parasites had never been found and which, therefore, were supposedly normal, an acute infection followed immediately. In fact, the downward trend in all the number counts in these superinfected monkeys and concomitantly the inefficacy of sporulation to produce any increase in the number of parasites after superinfection is markedly different from the acute increase in numbers in initially infected monkeys where comparable numbers were injected. To show this clearly, number counts at identical intervals are given for 5 initial infections in 3 spiders and 2 white throated monkeys (data from W. H. and L. G. Taliaferro, 1934) which were injected intravenously with enough parasites so that from 2 to 20 per 10,000 red cells appeared immediately afterwards in their blood (table 1). These number counts demonstrate decisively that parasites disappeared from the blood of these initially infected monkeys much more slowly than from the blood of superinfected monkeys. Thus, parasites in them decreased by a half (W21) or less in 44 hours whereas in the superinfected monkeys they decreased by three quarters or more (S3, 4, 16, 91, 364, W46) in the same length of time. Thereafter, the abundance of parasites in the controls differs strikingly from the paucity of parasites in the superinfected monkeys. Supplementary

* The superinfections in W46 and BS91 are also presented in this table for comparative purposes.

corroborative number counts on initially infected monkeys occur in the previously cited paper by us (1934).

Whenever 33 to 50 parasites could be examined during superinfection the regularity or irregularity of sporulation was ascertained (see footnotes † and ‡ in table 1). All of the observed sporulations were regular in 4 of the monkeys (3 sporulations in S3, 2 in S4, 6 in S16 and 13 in S91); but one of the 3½ sporulations in W46, S94 and S364 and both of the sporulations in S144 were irregular. These results are quite comparable to the results in the initially infected animals. Sporulation was regular in S113, but was irregular in 1 sporulation in W21 and S95 and in 3 sporulations in W141. In point of time, however, the irregularity, when it appeared, appeared sooner in the superinfected than in the initially infected monkeys.

In these natural and experimental initial infections and superinfections, 7 strains of *P. brasilianum* were used: 6 from red spiders (initial infections in S3, 4, 16, 91, 94, 144, 364, and W21 and 46 and superinfections in S3, 4, 16, 94, 144, 364 and W46) and one from a black howler (initial infection in W141 and superinfection in S91). Of these S144 was injected a first and second time with a mixture of the same 3 strains, S4 and 16 were naturally infected with one of the three strains with which they were superinfected and the balance were infected initially with a different strain from that used for their superinfection. An analysis of these data in conjunction with the number counts after superinfection discloses that the parasites in BS91 which was infected with a strain from a red spider and superinfected with a strain from a black howler did not disappear as soon as from the other monkeys which were infected and superinfected with strains from red spiders. Further work will be necessary to make sure whether this is an expression of a difference in the resistance of an individual monkey or whether it shows a less effective cross immunity between strains. In any case, between 6 (derived from red spiders) of the 7 strains an absolute cross immunity appeared to exist.

The more rapid rate of removal of parasites by the immune monkey, coupled with the fact that the spleen and liver which are rich in macrophages concentrated and phagocytosed parasites in a remarkably short time in superinfections of birds, led us to perform biopsies on spleens in 10 monkeys (6 white throated, 1 black and 1 red spider and 2 brown howlers) which had been superinfected from one hour to ten days. Nine of the monkeys were infected and superinfected with the same strain, although 1 white throated and 1 red spider may have been naturally infected prior to the experimental infection; the tenth mon-

key was superinfected with 1 of the 3 red spider strains used for its initial infection. Number counts from these spleens showed the same remarkable concentration and phagocytosis of parasites as has been described by Cannon and Taliaferro (1931) in avian work.

The very efficient immunity which latently infected monkeys possess against superinfection made it seem interesting to ascertain whether serum from such monkeys would protect against infection in normal monkeys as has so often been demonstrated in trypanosome infections. Under the conditions of our experiments it does not. For example, serum from naturally infected RS1 which had been infected for more than a year and had showed very few parasites in its blood for over a month was injected intraperitoneally into 4 brown howlers in doses of 1, 2, 3, and 3.4 cc. per 500 gm. monkey, respectively, along with a small simultaneous intravenous dose of parasites, whereas 4 other brown howlers were given an intravenous dose of parasites alone. Two days later parasites appeared in all the monkeys in thick films and the ensuing infections which lasted for from 10 to 20 days (animals died) were similar both as regards numbers and sporulation of the parasites. This experiment was repeated 4 times with serum from other latently infected monkeys with similar results. Serum-doses as large as 5 and 7 cc. per 500 gm. monkey were also ineffective when parasites and serum were injected intravenously simultaneously. In addition, 3 series were carried out after subjecting the parasites to *in vitro* contact with such serum for from 15 minutes to 1 hour and injecting the mixtures intravenously, but subsequent infections developed and were in no way different from those developing in monkeys which received only parasites and no serum. Furthermore, parasite counts from the tissues of these serum-treated animals seemed to be proportionately the same as from control animals whose normal infections had progressed for similar lengths of time. It might be objected that in these experiments the serum from spiders was tested in howlers, but this should not have invalidated the results inasmuch as monkeys when superinfected seemed to remove parasites originating from a different species of monkey as readily as from the same species.

In spite of these negative results we feel that there is an antibody basis for immunity to superinfection. In favor of such an hypothesis, there is the fact demonstrated in our earlier paper (Taliaferro, W. H. and L. G., 1934) and in the present one that the intense immune reaction at the time of the crisis is often associated with a number of degenerating forms which are probably the result of some humoral principle. The immunity to superinfection has been shown in many

cases to be highly specific (see, for example, investigations on avian malaria by Gingrich, 1932, simian malaria by Mulligan and Sinton, 1933, and human malaria by Boyd and Stratman-Thomas, 1933, and review in Thomson, 1933) which is difficult to explain except on the basis of a specific opsonizing antibody (see Taliaferro, 1932). Although of low titer and inconstant, precipitins and complement-fixing antibodies have been demonstrated in human malaria (see review in Taliaferro, 1929) which are probably closely related to, or identical to the postulated opsonin. In avian malaria we (Taliaferro, W. H. and L. G., 1929) were unable to find protective antibodies, but Findlay (in Thomson, 1933) maintains that with proper technique they can be demonstrated and curative and protective antibodies have been described in man (see review in Thomson, 1933). In brief we feel that the immunity to superinfection in malaria is primarily cellular, but is mediated by an opsonizing antibody which is produced locally in the organs most directly concerned in removing the parasites, i.e., the spleen, liver and to a slight extent the bone marrow, but which when diluted by the blood is of too low a titer to be evident in protection experiments.

Conclusions.

1. (*a*) Immunity to superinfection of *P. brasilianum* has been demonstrated by the rapid decrease and disappearance of parasites from the blood of latently infected monkeys (12 monkeys) when injected intravenously with large numbers of parasites of the same strains or combination of strains as compared with the increase and accumulation of parasites in the blood of uninfected monkeys when injected similarly with large numbers of parasites for the first time.

(*b*) This immunity is effective immediately after the initial infection has abated and lasts for more than a year (as long as tested).

2. (*a*) Effective cross immunity to several strains of *P. brasilianum* has been demonstrated by the same technique since 5 monkeys showed a rapid disappearance of the superinfected parasites when initially infected with one strain and superinfected with another strain, both originally derived from different red spiders of the species *Ateles geoffroyi*, while one monkey showed a less rapid but eventual disappearance of the superinfected parasites when initially infected with two strains derived from red spiders and superinfected with a strain from *Alouatta palliata inconsonans*, the black howler.

(*b*) This cross immunity is effective immediately after the initial infection has abated and lasts at least a year (as long as tested).

3. All stages of the asexual cycle are removed since the parasites begin to disappear immediately after superinfection no matter at what stage they are when introduced.

4. Serum from monkeys with latent malarial infections is without protective action (i.e., infection ensues) when injected into uninfected monkeys either simultaneously with parasites in the same or different sites or after 15 minutes to 1 hour *in vitro* contact with them.

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