PASSIVE IMMUNITY IN AVIAN MALARIA*

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Much has been written on immunity in malaria during the past decade. Indeed, it is probably proper to say that the most important additions to our knowledge of malariology have been in this field, and perhaps also in that of the life history and biological relationships of the malaria organisms. We are still nevertheless in the position of being compelled to admit that what is known as to the mechanism of immunity in malaria may be only a fraction of the whole story. We believe that we can say with assurance that it is primarily a cellular mechanism, based quite largely on the activities of the macrophages of the liver and spleen. We know also that it is highly specific, even strain-specific. Just at present it is this last fact which seems to be most difficult to account for, and it is here that certain factors present in the serum may come in.

Coggeshall and his associates (1937, 1938) have been able to show that in monkeys such factors are present. They have demonstrated that *rhesus* monkeys may be protected against infection with *Plasmodium knowlesi* by repeated injection of immune serum, that there is a relationship between the amount of immune serum and the amount of protection conferred, and that complement-fixing antibodies are produced as well as agglutinating and protective antibodies. Sotiriadès a number of years ago claimed that the injection of serum from latent cases of human malaria had a beneficial therapeutic effect, which would indicate that such substances are also present in human immune serum. Efforts to show that the serum of chronic cases of avian malaria contained protective substances have also been made, but in no case have very definite results been obtained. Taliaferro (1931) found slight evidence of such protection, but he could not obtain it consistently enough to convince himself that it was more than a chance occurrence. Hegner and his coworkers (1938, 1939) attempted to confer passive immunity to *Plasmodium cathemerium* by injecting birds with serum from acute cases, chronic cases, and also with emulsions of splenic tissue from such infections, but obtained no very positive results. They believed, however, that they did get slight protection, as measured by alleviation of symptoms and pathology in some of the birds which received dried serum or dried splenic tissue from acute cases. But there was no effect on the parasite number. Brown (1933) and Findlay and Brown (1934) believed

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that experiments carried on by them showed that there was some substance in the serum of birds recovering from an acute infection, or carrying a chronic infection, which exerted a protective action when injected into other birds inoculated with malaria. However, this substance was non-specific in its effects, and they thought it was correlated with a reduction in the electric charge carried by the red cells. Others, as for example Lotze (1931, 1932), have attempted passive immunization, but without any success.[1]

The present study was undertaken in the belief that the demonstrated general similarity of the malaria plasmodia in other respects made it likely that the host reaction in avian malaria would prove like that in monkey and human malaria if investigated with sufficient care. It was hoped, too, that facts could be obtained which would help in explaining the highly specific nature of the immune response in malaria. The results have been briefly reported earlier (Manwell and Goldstein, 1938).

Materials and Methods

Plasmodium circumflexum was selected as the species to be used, because we had already done much work on it in connection with determining the amount of protection conferred by different strains against others, and thus its behavior was already well known from the study of some hundreds of cases. It is also particularly good material for a study of immunity because the parasite level in canaries during the chronic infection is usually very low. Hence when resistance to the parasites is broken down by one means or another, one may be reasonably sure it is not a naturally occurring relapse. It is also an indication of a high degree of immunity, and therefore suggests the formation of very effective antibodies.

The origin of the two strains used is given in detail in a previous paper (Manwell and Goldstein, 1939). The two strains of which most use was made are designated as "A," which was isolated originally from a song sparrow caught in Syracuse on Apr. 26, 1935, and "E," which was obtained from a red-winged blackbird trapped by Dr. Carlton Herman at the Austin Ornithological Station, North Eastham, Massachusetts, in the summer of 1936. These strains seem to be identical morphologically, but they differ immunologically, since the latter is able to break down the resistance conferred by chronic infections of the former quite regularly.

Female canaries were used throughout the study, and infections were transferred by injection of citrated blood, usually from acute cases.

[1] Several papers have appeared recently, among them those of Causey (1939) and Herman and Goldfarb (1939), which tend to show that the spleen is less important in the mechanism of immunity to malaria than has been thought. To us it seems that this work has significance, not as proving the normally slight importance of the spleen in malarial infections, but as indicating that its rôle in immunity is shared by other organs, such as the liver, which if necessary may take over the entire task.

The immune serum was secured from chronic cases which had been previously given from four to six injections of parasites containing from 1,000,000 to 6,000,000 infected cells to raise the titre of protective factors, as demonstrated by Coggeshall and Kumm (1938) in monkeys carrying infections of *Plasmodium knowlesi* and by Redmond (1939) in avian malaria due to *Plasmodium relictum* and *cathemerium*. The blood from a number of such birds was pooled, since relatively large quantities were required. It was

TABLE I

*Summary Table of Passive Immunity Experiments**

Experiment	Cases		Serum administered		Sources and quantities of inoculum					Degree of protection†		
	Treated	Controls	Before infection	Simultaneously with and after infection	Serum‡ Strain	Parasites Strain	Number of parasites per bird at infection§	Serum inoculations per bird	Average amount serum per injection	Complete Number of cases	Partial Number of cases	None Number of cases
									mg.			
A	3	2	x		A	E	6000	9	93	3	0	0
B	3	3	x		E	A	1,000,000	9	132	2	0	1
C	3	2	x		E	E	6000	9	93	3	0	0
D	3	3	x		A	A	80,000	8	133	1	0	2
E	3	3	x		E	E	30,000	8	162	2	0	1
F	4	2		x	A	E	3 cases = 50,000 1 case = 150,000	8	97	0	4	0
G	4	2		x	A	A	3 cases = 90,000 1 case = 250,000	8	98	3	1	0
H	3	3		x	A	A	90,000	7	133	1	0	2
I	3	3		x	E	E	8000	8	155	3	0	0
J	3	3		x	E	A	2,500,000	7	143	0	2	1
Totals...	32	26	15 cases	17 cases	17 cases = A 15 cases = E	16 cases = A 16 cases = E		Average = 8.1	Average = 123.9	18	7	7

* See graphs for details of each case.

† Complete = no parasites observed; no infection upon subinoculation.
Partial = experimental curve was lower than the lowest control curve.
None = experimental curve higher than the lowest control curve.

‡ All immune serum placed in the incubator for 30 minutes at 56°C.

§ All inoculations intramuscular except Experiments B and H which were subcutaneous. All parasites mixed with immune serum of the kind used in the experiment then placed in the incubator for 30 minutes at 37°C. Controls treated similarly but with normal serum from clean birds.

obtained either by bleeding from the leg vein, or by decapitating the bird, and clotting was prevented by mixing with citrated saline, or saline to which heparin had been added. The proportion of whole blood to saline was usually 2 to 1. After centrifuging, the serum was pipetted off and heated for a half hour at 56°C. It was then incubated with the inoculum of parasites for 30 minutes at 37°C. in all cases at the time of the initial infection. The controls in such experiments were inoculated with parasites similarly

incubated with normal serum. Serum administration was in various ways—intravenous, subcutaneous, and intramuscular. The number of treatments varied between seven and nine. Details are given in Table I, and also in Charts 1 to 4, in which case histories are given. In general, the treated birds can be divided into two groups, those which received serum before infection, and those which received it simultaneously with and after infection.

The numbers of parasites in the inocula were determined by finding the ratio of parasitized cells to the unparasitized erythrocytes on a stained slide, and calculating from this the number probably present in the quantity infected. This method undoubtedly gives only an approximation, but since it was used in all the experiments it at least makes them comparable.

The degree of protection conferred by the treatment was measured both in terms of the delay in the length of the incubation period, and by making counts of the parasites observed each day in a given number of fields. From this the number of parasites per 10,000 red cells was calculated and charted as seen in Charts 1 to 4. A number of birds never showed parasites and these were tested for the presence of a latent infection by subinoculation into clean birds, and subsequent reinoculation. The only exception to this was in a few cases which died, and these were autopsied and the volume and dimensions of the spleen recorded.

After it became clear that passive immunity could often be conferred by the injection of immune serum, a large number of chronic cases from which considerable quantities of blood had been taken were injected with heavy doses of parasites soon thereafter. They were then followed for 2 weeks to see whether their immunity had been at all weakened by the withdrawal of so much serum. The interval which was allowed to elapse before superinfection varied from one-half day to 8 days. The dosage of parasites also differed considerably, and no effort was made to determine it very accurately. In about half the cases 30 c.mm. of blood containing about 25 parasites per field was given, so that each bird received approximately 31,500,000. The dosage in the other cases was smaller.

Some experiments were also carried out to determine the effect of the injection of serum, both normal and immune, and of physiological saline upon the spleen of clean birds. The volumes of the individual injections, and their number, were roughly equal to those used in the passive immunity experiments. The spleens of all such birds were examined at the conclusion of the experiment as in those cases in which serum therapy had apparently prevented infection.

To determine whether agglutination was induced by immune serum, a suspension of parasitized cells was mixed with such serum, after treatment as outlined above, and allowed to stand for 2 hours. It is essential that the antigen contain at least 2000 parasites per 10,000 red cells, and success is more likely to be had if the greater number of parasites are nearly mature. Gentle mixing for 15 minutes facilitates the reaction. Not less than one part of antigen to five parts of serum should be used.

RESULTS

1. Passive Immunity.—

The accompanying graphs illustrate the results obtained in each series of experiments. They are really to be considered from three angles: (*a*) the

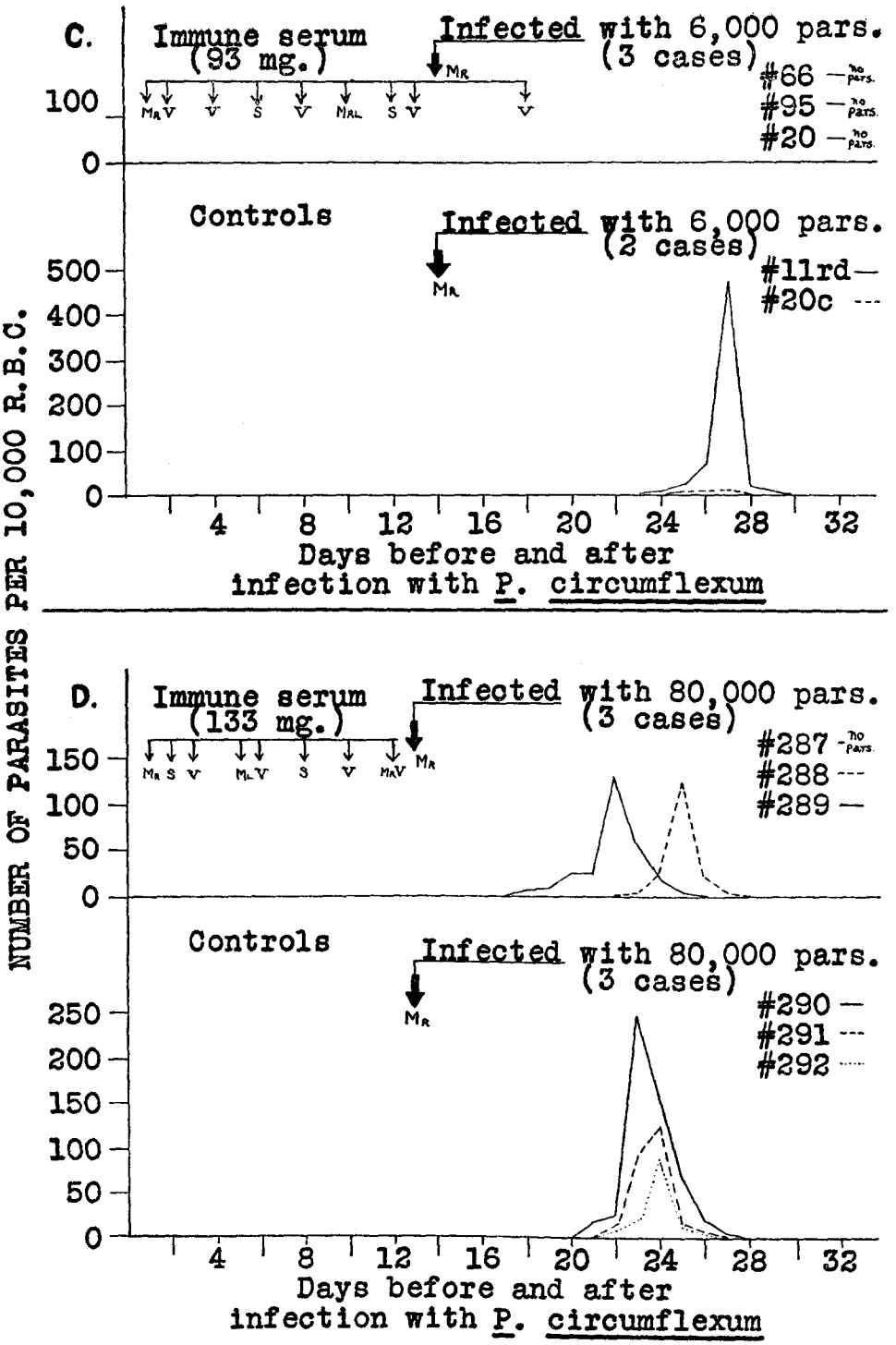

CHART 1. Experiment C, E serum given before E parasites. Experiment D, A serum given before A parasites. V, intravenous inoculation. S, subcutaneous inoculation. Mr, intramuscular inoculation in the right breast. Ml, intramuscular inoculation in the left breast. Mrl, intramuscular inoculation in both breasts.

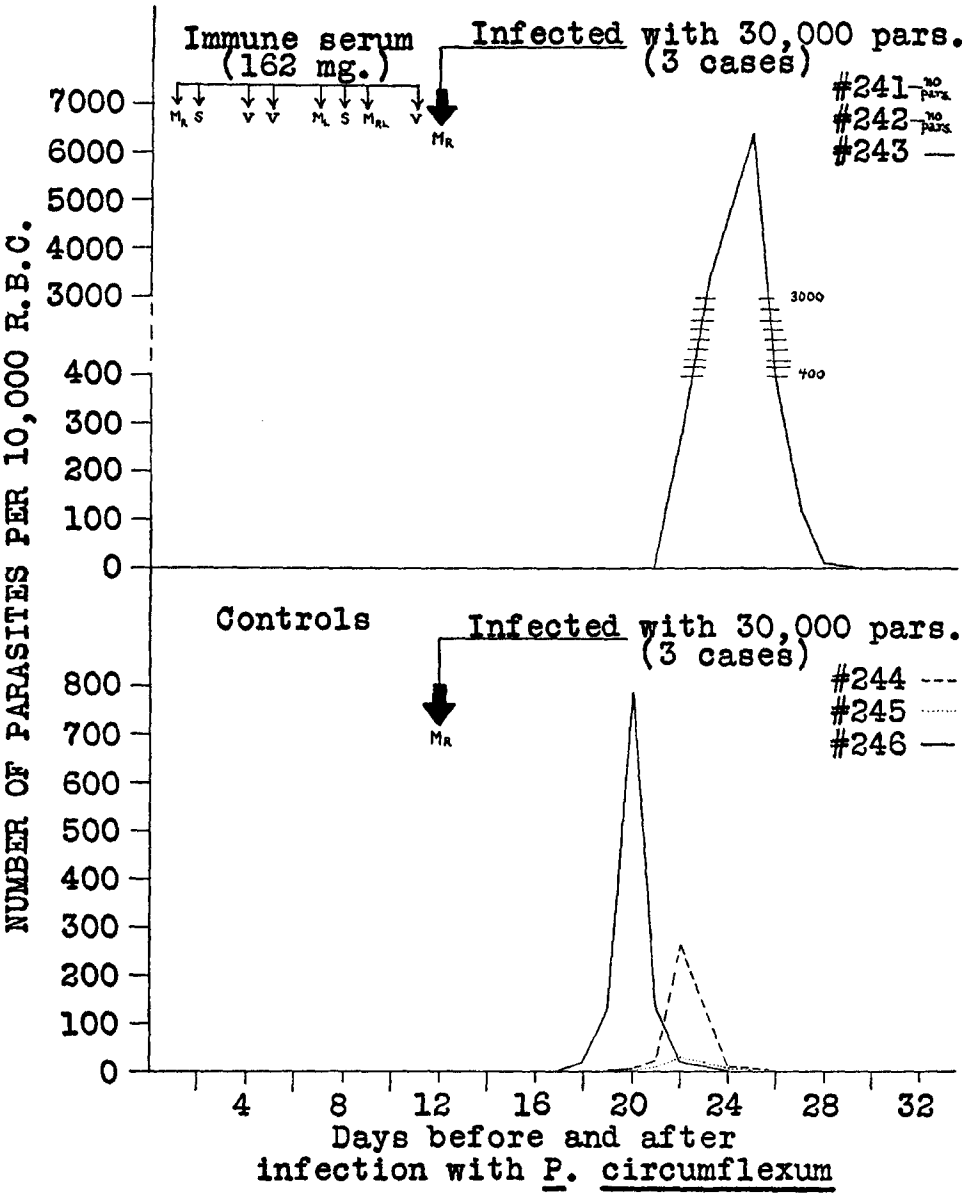

CHART 2. Experiment E, E serum given before E parasites.

effect of immune serum administered before infection, (*b*) the effect of serum therapy when started at the time of infection, and continued for some time thereafter, and (*c*) the problem of strain specificity.

There is no question as to the possibility of conferring a temporary passive

immunity. This can be most easily done when the immune serum is administered before inoculation with parasites. Of fifteen cases so treated eleven developed no infections at all, and four were apparently not protected. The criterion of infection was that infections, if any resulted, must be of lesser severity than that of the mildest infection observed in any of the controls. The degree of severity was measured in terms of the numbers

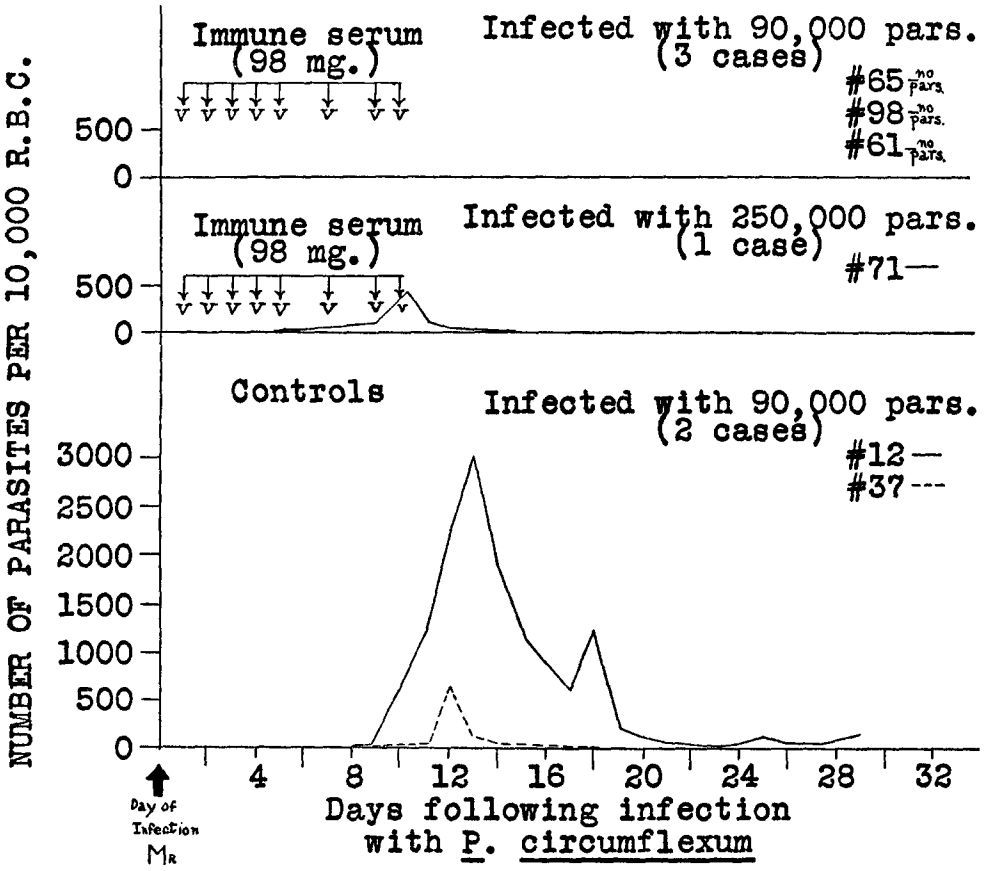

CHART 3. Experiment G, A parasites and A serum given simultaneously.

of parasites and length of infection; symptomatology and pathology were not considered. It will be noted too that some of the birds were given relatively large doses of parasites, although on the whole the dosage was rather small since it was felt that if immunity was present it could be better detected under these circumstances. The protective effects can hardly be ascribed to direct action of the immune serum upon the parasites, for it

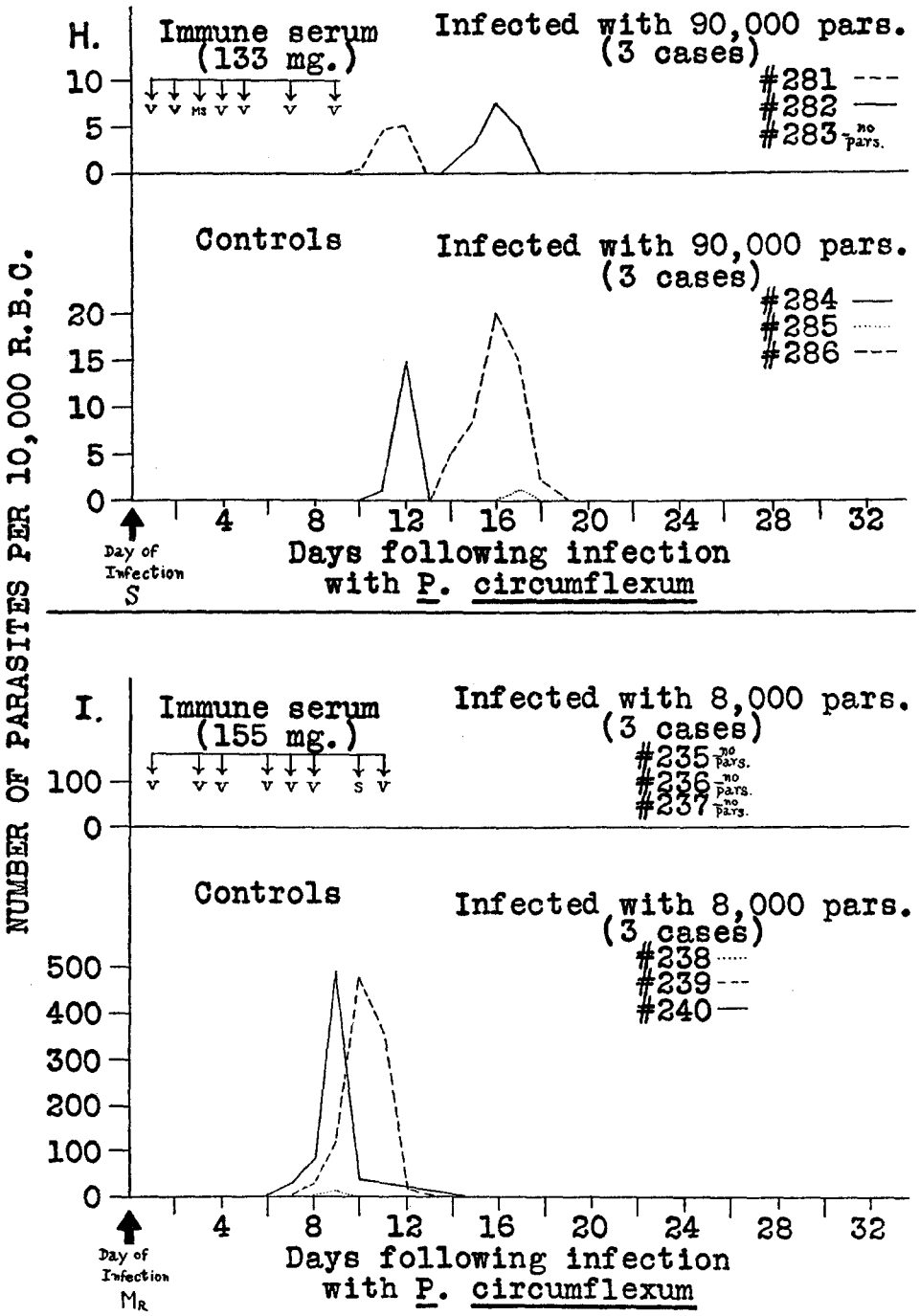

CHART 4. Experiment H, A parasites and A serum given simultaneously. Experiment I, E parasites and E serum given simultaneously.

is a well known fact that the blood of chronic cases is infectious to clean birds, even though it usually contains very small numbers of plasmodia. These plasmodia have, of course, been incubated with the blood in which they are contained in the infected bird, so to speak, for a very considerable interval prior to their inoculation into a clean bird. It is true that infections produced in this way are frequently not very severe, but it is fully as likely that any such differences in severity are due to the small numbers of parasites in such blood as that any immune substances in the serum are concerned. And it is quite certain that the longer incubation periods observed under these circumstances may be so explained.

The results of serum therapy given simultaneously with infection and thereafter were less striking, but nevertheless convincing. Of seventeen birds so treated seven were completely protected, seven partially protected, and three showed no indication of any protection at all. Indeed three of the treated birds died, one of them after an overwhelming infection. One of these three, however, is listed as "partially protected" because it showed very few parasites, even at the height of the acute stage. Death occurred on the 12th day after inoculation, when it seemed to be recovering.

All of the controls, in both series of experiments, developed ordinary infections with a single exception. In this case the parasites were inoculated subcutaneously, and even though each bird was given approximately a million of them, the infections developed very slowly, which suggests that the mortality of the parasites when injected in this manner must be very considerable. The exception occurred in Experiment B.

These experiments do not throw much light on the question of strain specificity. The two strains of which most use was made, strains A and E, appear to differ immunologically as has already been stated. Chronic infection with E seems to protect against A but not the other way about. When the results of therapy with the homologous and heterologous serum are compared, however, no very significant differences in efficacy appear to exist. E serum gave complete protection against A parasites in one case, partial protection in three, and none at all in two. The results when A serum was used against E parasites were slightly more favorable. Three birds developed no infections, four developed milder ones than the controls, and there were no cases without protection. Thus we have a total of four birds completely protected, seven partially protected, and two not protected at all.

When A serum was used against parasites of the same strain, five cases never showed evidence of infection, one developed a mild infection, and four developed typical infections. Serum from chronic E cases seemed

more effective. Eight of the birds which received it never showed parasites, there were none only partially protected, and only one which developed an infection as severe as those of the controls. The totals are, therefore, thirteen cases completely protected, one partially protected, and five not protected at all, when parasites and serum of the same strain are used. On the whole, it appears that immune serum is more effective when used against a homologous strain of parasites, than when the two are heterologous, but it would require a larger number of cases to determine this point with certainty. Perhaps it is significant that serum from chronic cases of E was more effective against parasites of this strain than when any other combination of parasites and serum was used, for on the whole, this strain seems to develop a more effective immunity than any of the other six strains which have been studied (Manwell and Goldstein, 1939).

2. Results of Superinfection after the Withdrawal of Large Quantities of Serum.—

The results of this part of the experiment were completely negative as far as any evidence of breakdown of the resisting mechanism was concerned. Although the experiments which have already been described showed convincingly enough that protective substances are present in the serum of chronic cases, in no case was the resisting mechanism perceptibly weakened by the loss of relatively very large amounts of serum. Large doses of parasites were promptly disposed of, and there were no relapses thereafter, unless the observing of a single parasite in smears from three of these cases should be so interpreted. It is, therefore, clear, as has been repeatedly shown by other workers with other species of malaria, that in *Plasmodium circumflexum* infections also, the defensive mechanism is presumably primarily cellular. Large quantities of immune serum, and (in general) relatively small doses of parasites are required to demonstrate any protective effect, and the withdrawal of very considerable quantities of such serum neither results in relapse, nor in a perceptible loss of ability to cope with large numbers of introduced parasites. Such protective substances as are present in immune serum must be there in rather low concentration and of relatively low potency if they have any direct action on the parasites, or it may be that they have some function in connection with the stimulation of the cellular defensive mechanism.

3. Effect of the Injection of Normal Serum and Physiological Saline upon the Spleen of Normal Birds.—

After it had been observed that the spleens of birds which had been treated with immune serum were much larger than the normal, even when there was no evidence of malarial infection, it was decided to treat a number

of birds with normal serum and others with physiological saline to see whether the splenomegaly was dependent on immune factors as we had suspected, or whether it was entirely non-specific. When such birds were autopsied at the conclusion of the course of injections, we were much surprised to find that they exhibited the same degree of splenic enlargement that those treated with immune serum did, and this appeared to be true irrespective of whether serum or physiological saline had been used. What the explanation of this is we do not yet know. Possibly it is merely the result of adding relatively large quantities of fluid to that already present in the circulation, although one would expect that any effect arising from that cause would be very transitory. We have not yet completed histological studies to see what cellular changes may have occurred, if any.

4. The Question of Agglutination.—

Since agglutinating antibodies have been found in the blood of *rhesus* monkeys suffering from a chronic infection with *Plasmodium knowlesi* (Eaton, 1938) it seems logical to suppose that they also occur in infections with other species of plasmodia and in other species of hosts. With the demonstration that the serum of malaria-infected birds contains protective factors it was therefore examined for agglutinating properties also, and it was found that agglutination could be quite easily observed with a suspension of blood heavily parasitized with *Plasmodium circumflexum* and immune serum taken from chronic cases. When such blood was mixed with normal serum or with the serum from other acute cases, no agglutination was observed. Usually agglutination occurred in about 2 hours. Agglutination was always microscopic and could not be observed macroscopically.

DISCUSSION

Certain facts of interest seem to stand out when the results reported above are examined in the light of similar work reported by others. One is the apparent difference which exists between different species of plasmodia with respect to their ability to infect, and perhaps also in the potency of immune serum. For example, Coggeshall and Eaton (1938) found that the minimum infective dose for *rhesus* monkeys when injected with *Plasmodium knowlesi* was between 1 and 10 parasites. Protective treatment with immune serum was efficacious in preventing infection with doses containing as many as 10,000 parasites in some cases, and the severity of the infection seemed mitigated even when some considerably larger doses were used. *Plasmodium circumflexum* seems to be roughly comparable as far as the potency of immune serum is concerned (although when the difference in the size of

rhesus monkeys and canary birds is considered, it may appear that the serum is less effective), but our experience indicates that the minimum infective dose must be considerably greater, although the mode of inoculation also seems to make a good deal of difference. So far, we have not been able to secure infection with single parasites. This has been done by Stauber (1937), however, using *Plasmodium cathemerium.*

Our results also emphasize the fact that whatever immune substances are present in serum must be there in relatively very small concentration. The fact that the withdrawal of very large amounts of such serum (even up to 500 c.mm., which must be close to half of the total quantity present in the circulation) does not result in relapse, or perceptibly lower the ability of the bird to dispose of large numbers of added parasites, suggests either that these substances are very rapidly regenerated, or as seems more likely, that they do not constitute a very important element in the defense mechanism.

It is also rather surprising to find so wide a variation in the relative efficacy of the immune serum, in view of the fact that the chronic cases from which it was taken appeared to exhibit a strong immunity. Probably the explanation of this fact lies in the mode of action of the serum, which, as suggested above, is presumably indirect. If something in the serum operates to stimulate the cellular defense mechanism, we could explain the variable efficacy of serum therapy in terms of variations in the sensitivity of this mechanism.

Something also needs to be said as to why so many efforts which have been made in the past to demonstrate protective properties of the serum in avian malaria have failed, or have given inconclusive results. We believe that the explanation lies largely in two facts: (*a*) the serum-treated birds have been inoculated with too large numbers of parasites, so that whatever protection there may have been has not been perceptible, and (*b*) the immune serum which is obtained from ordinary chronic cases contains an insufficient concentration of protective substances. Unless previous superinfection is resorted to in order to raise the immune titre, such substances would be very difficult to detect, unless very large quantities of serum were used. Or it might be possible to secure more potent serum from recently recovered cases, when the degree of immunity seems to be highest. We showed in work done some years ago with *Plasmodium cathemerium* (Manwell, 1929) that in this species relapses were least frequent in a period of from 3 to 5 months after the initial infection.

Another angle of the problem which is of interest is the question as to whether immune serum would operate in mosquito-induced infections as it

seems to do in those produced by blood inoculation. The fact that antimalarial drugs appear to be less effective against sporozoite infections than against others suggests possibly that the same thing might be true of immune serum.

SUMMARY AND CONCLUSIONS

The effect of therapy with immune serum has been studied in thirty-two cases of *Plasmodium circumflexum* infection, all of them produced by blood inoculation. Eighteen of these cases never showed parasites, and seven others developed infections which were definitely milder than those of the controls. The therapeutic serum was in all cases obtained from chronic cases which had previously been superinfected to raise the immune titre. It seems justifiable to conclude that:

1. Passive immunity can be conferred in avian malaria, at least when caused by *Plasmodium circumflexum*, just as it can be in certain types of monkey malaria, and perhaps in human malaria as well.

2. Whatever the nature of the protective substances present in the serum of chronic cases may be, they are present in very low concentration. Their concentration can be raised by superinfection, however. These substances may be strain-specific or species-specific, but the results of these experiments do not give any clear-cut answer to this question.

3. Serum therapy previous to infection seems to be more effective than when given afterward.

4. The administration of normal serum or even of physiological saline in a dosage comparable to that employed with the immune serum used in these experiments produced similar macroscopic changes in the size of the spleen.

5. Agglutination of cells parasitized by *Plasmodium circumflexum* when mixed with immune serum was observed.

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