

Subtitle: Immunization of Rats Against Plasmodium Berghei with Plasmodial Homogenate, Carboxymethyl-cellulose (CMC) Bound Homogenate, and CMC-Homogenate Followed by Administration of "Immune" Gamma Globulin

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The possibility of producing effective active immunization against malaria has occupied the attention of many investigators for more than fifty years. While there is no single recent comprehensive review of the many attempts to induce a protective immunity, reference can be made to the pertinent papers in the Proceedings of the International Panel Workshops (1964, 1966).

Most attempts at immunization have employed killed plasmodia. The success with this form of antigen has, in general, been highly variable but this is not surprising considering the many different methods of antigen preparation, immunizing schedules, species of Plasmodium and hosts used. There is indication that immunization with adjuvant-antigen mixtures afford better protection than antigen alone (Freund et al, 1945, 1947, 1948). Successful immunization of monkeys was obtained by Targett and Fulton (1965) by use of P. knowlesi-Freund's adjuvant. Intramuscular injection of the mixture seemed to obviate the untoward effect usually attendant upon the use of Freund's adjuvant. Zuckerman et al (1965) noted a partial protection of rats against P. berghei after they had been given a series of immunizing doses with cell-free homogenates of parasites. These authors found that the only difference between inoculation with or without adjuvant was that a single dose of the adjuvant-antigen was apparently as effective as three doses of the antigen alone.

Moroz and her colleagues showed (1963) that the immunogenicity of viper venom neurotoxin was enhanced when bound to soluble carboxymethyl-cellulose (CMC). This promising technique had not been applied to parasite immunology and it was thought of interest to determine whether a similar effect could be obtained with malaria homogenate bound to CMC. This present communication gives the results of P. berghei homogenate CMC immunization and the effect of "immune" gamma globulin given to antigen-CMC immunized rats prior to challenge.

Methods

White rats weighing between 200 and 225 grams were used in these experiments. The advantage of using the white rat is that unlike the mouse, fulminating infections are not generally produced, thus any partial immunizing effect is more likely to be detected.

The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

Antigen was prepared from the pooled blood of heavily infected mice. Parasites were freed from the erythrocytes by the dextran-saponin method of Spira and Zuckerman (1966). Homogenate of the washed plasmodia was then made by use of a Hughes press. Micro-Kjeldal analysis was performed for all antigen preparations which were then diluted in 0.005M phosphate buffer (pH6.8) to contain between 6.5 to 7.5 mg protein per ml. The extract was divided in half and to one portion was added 40 mg CMC per ml according to the method of Moroz et al (1963).

In the first experiment, of 60 rats one group of twenty was inoculated with 0.1 ml antigen intramuscularly and another group of 20 given 0.1 ml antigen-CMC by the same route. A group of 20 rats were set aside as controls. Three immunizing doses spaced two weeks apart were given. Two weeks after the last inoculation all animals, including the 20 controls, were challenged by intraperitoneal inoculation with approximately 68×10^6 parasites in 0.1 ml mouse blood. Thin blood films of each animal were made daily

for thirty consecutive days. These films, made by an experienced technician were with of a high degree of uniformity. The assessment of immunizing effect generally followed that used by Zuckerman et al (1965). The total number of parasites in 100 thin oil-immersion fields were counted and from this the first day of patency, the number of plasmodia at peak parasitaemia and the day at which peak parasitaemia occurred were noted. While it is recognized that there are inherent deficiencies in this method of estimating parasitaemia it is probably no less accurate than more complicated enumerative techniques. Given slides prepared with reasonable uniformity it is felt that this method gives an acceptable means of assessing the course of the parasitaemia.

At the end of experiment one, all the rats were reinoculated with 0.1 ml heavily infected mouse blood and killed two weeks later. The globulin from the pooled sera of all sixty animals saturated ammonium sulfate method of Kendall described by Kabat and Mayer (1964). Paper electrophoresis showed that the fraction so obtained was almost entirely gamma globulin with a small amount of beta globulin.

In the second experiment, of forty white rats, twenty were immunized with CMC-antigen as in the first experiment. Two days prior to challenge these animals and ten non-immunized rats were intraperitoneally inoculated with approximately 15 mg of gamma globulin on each of two successive days. These rats along with ten controls were then challenged with approximately the same number of parasites used in experiment one and the course of infection studied exactly as in that experiment.

Since both the antigen and challenging parasites were derived from mouse blood the possibility existed that some of the immunizing effect might be due to antibody produced to mouse erythrocyte material. In order to determine whether this factor was present 20 rats were immunized with stroma prepared from uninfected mouse blood. This material was obtained in the same manner as that for an equal amount of infected erythrocytes. When these rats were challenged following the completion of the immunizing course it was found that the prepatent period was extended for an average of 0.5 days as compared to the controls. The average peak parasitaemia attained by both groups was not significantly different.

Results

The results are summarized in Table 1. Since Students t-Test showed no significant difference in parasitaemias between the control groups of experiments one and two all results are listed together for convenience of comparison. Both antigen alone and antigen-CMC produced an immunizing effect. However the degree of immunity produced by antigen-CMC seemed to be of a higher order than antigen alone. The average parasitaemia of the antigen group was approximately one half that of the controls and that of antigen-CMC one third. The number of days of prepatency and peak parasitaemia of both immunized groups were greater as compared to the controls. The median and mode of the prepatent period for the antigen-CMC group was slightly longer (4 days) than that of the antigen group (3 days).

The inoculation of the "immune" gamma globulin had no effect on the intensity of parasitaemia ultimately produced. However, the parasitaemia of those rats given antibody developed more slowly, as reflected by median-mode days of peak parasitaemia than that of the controls. That there was a combined effect of active immunization followed by passive transfer of antibody was evidenced by the considerably longer median-mode prepatent period (7 days) and day of peak parasitaemia (10.9 days) of the antigen-CMC-gamma globulin group than of the group immunized with antigen-CMC. While the average peak parasitaemia, was the same for both groups it is of interest to note that only in the group given antigen-CMC-gamma globulin were there any rats (3/20) completely protected for the entire 30 day observation period.

During the thirty day observation period, the rats in all groups experienced a number of parasitaemic recrudescences which were generally of a progressively diminishing intensity. There is some evidence, despite a wide variation in parasite densities from rat to rat within any one group, that immunization affected the intensity of the parasitaemia at least at the first recrudescence. In experiment one

the average density at the first recrudescence for the controls was 753/100 thin oil-immersion fields, whereas for the group immunized with extract only it was 479 and for the group given extract-CMC it was 290. There was little difference in the nature of the first recrudescence between the groups given antigen-CMC and antigen-CMC-gamma globulin.

Discussion

The results presented in this paper indicate that immunization with antigen bound to CMC produced a significantly superior immunity to that of antigen alone. That the homogenate antigen also gave some partial protection by lowering the parasitaemia and extending the prepatent period is in agreement with the findings of Zuckerman et al (1965). In contrast to the use of antigen-CMC, these authors reported that there was no advantage to the use antigen-Freund's adjuvant other than shortening the course of immunization. Immunization with antigen-CMC seems to be worthy of further trial and experiments with primate malaras are planned.

That administration of antibody alone slowed the progress of the parasitaemias but did not ultimately effect the ultimate course of infection confirms the recent work of Briggs et al (1966). There was an additive effect of active immunization plus "immune" gamma globulin in that the prepatent period was considerably increased by this treatment. It is felt that this line of investigation also deserves further study using larger amounts of gamma-globulin and with other host-malaria parasite systems.

Table 1. The effect of immunization with P. berghei extract, extract-CMC, and extract-CMC followed by administration of "immune" gamma globulin.

Group	1st Day Parasitaemia			Peak Day Parasitaemia			Peak Parasitaemia	
	Median	Mode	Range	Median	Mode	Range	Arith. mean	95% Confidence Limits
Controls n=30	1	1	1-7	5	5	1-9	1,230	1,050-1,410
Gamma globulin n=10	1	1	1-6	8	8	6-10	1,473	1,222-1,724
Antigen n=20	3	3	2-8	7	6	5-12	635	542-728
Antigen-CMC n=20	4	4	2-7	7	7	6-12	403	323-483
Antigen-CMC gamma globulin n=20	7	7	4-00*	10	9	8-100*	440	335-545

* Three animals did not develop parasitaemia during the 30 days followed.