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Circulating immune complexes in rodent and simian malaria

B. RAVINDRAN, R. R. SHARMA, B. K. SHARMA and
Q. Z. HUSSAIN
Department of Biochemistry/Immunology, National Institute of Communicable Diseases, New Delhi 110 054

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Abstract. The circulating immune complexes have been detected in the sera of albino rats infected with *Plasmodium berghei* and rhesus monkeys infected with P. *knowlesi* by (i) quantitative cryoprecipitation assay and (ii) polyethylene glycol assay. In the rodent model, the levels of circulating immune complexes increased during infection and decreased considerably in the post-infection period. In the simian system, high levels were detected during peak parasitaemia. Polyethylene glycol precipitate obtained from the sera during acute *P. knowlesi* infection when analysed by Immunoelectrophoresis was found to contain (i) monkey IgG, (ii) four other components of monkey plasma, (iii) two components of normal monkey erythrocytes and (iv) antigen(s) of *P. knowlesi.*

Keywords. Circulating immune complexes; rodent malaria; simian malaria; *Plasmodium berghei;Plasmodium knowlesi.*

Introduction

Immune complexes have a well established role in causing.tissue damage, and are also known to suppress the humoral and cellular immune responses of the host against various antigens (World Health Organisation, Technical Report Series, 1977). Soluble circulating malarial antigens have been demonstrated in rodent, simian and human malaria models (McGregor *et al.,* 1968; WHO, Technical Report Series, 1977). In human malaria, the antigens were shown to circulate either free or bound in immune complex forms for several weeks after clearance of parasites by chemotherapy (Houba and Williams, 1972). It has been suggested that malarial immune complexes trigger a pathogenic sequence in which other mechanisms, possibly autoimmune are involved later (Houba, 1975). The presence of circulating immune complexes (CIC) in experimental malaria in mice has been reported (June *et al.,* 1979; Contreras *et al.,* 1980). Although June *et al.,* (1979) obtained indirect evidence for the presence of specific malarial antigen and antibodies in circulating immune complexes in the sera of mice injected with *Plasmodium berghei,* further confirmation that malarial antigens are also present in

Abbreviations used: PEG, poly ethylene glycol; PBS, phosphate buffered saline.

the circulating immune complexes is still awaited. There are no reports to date*, regarding the detection and chracterization of circulating immune complexes in rhesus monkeys infected with *P. knowlesi.*

In the present study, circulating immune complexes have been detected in the sera of albino rats infected with *P. berghei* and rhesus monkeys infected with *P. knowlesi* by (i) quantitative cryoprecipitation assay and (ii) polyethylene glycol (PEG) assay. Further, the circulating immune complexes which appear during the acute phase of *P. knowlesi* infection have been analysed for their components by Immunoelectrophoresis.

Materials and methods

Animals

Male rhesus monkeys (*Macaca mulatta*) weighing 3 to 4 kg were quarantined and screened for tuberculosis. The animals were kept under quarantine in our animal house for 30 days and were tuberculin-tested before use. A natural diet of fresh fruits, vegetables, nuts and soaked pulses was given during the day. Water was provided *ad libitum.*

Male albino rats (Wistar strain) weighing 150-180 g were used in the study. Albino rabbits of both sexes weighing 1-1.5 kg were used for raising antisera. The animals were provided with a standard diet (wheat flour, ground nut, olive oil, black gram (*Phaseolus mungo*) NaCl and water *ad libitum.*

Strain of parasites

Plasmodium knowlesi W1 variant, was obained from Guy's Hospital Medical School, London, UK and cryopreserved in 30% glycerol at —70°C. (One volume of infected erythrocytes +2 volumes of 30% glycerol in 0.15 M phosphate buffered saline, pH 7.2). The frozen parasite stabilate was revived by passaging the material intravenously into a fresh rehesus monkey which was later used as a donor of parasites. This strain of *P. knowlesi* was uniformly lethal to rhesus monkeys causing death within 5 to 6 days after patency. The strain of *P. berghei* used in this study was obtained from Prof. P. C. C. Garnham, London School of Hygiene and Tropical Medicine, London, UK. This strain of *P. berghei* was not highly lethal to adult rats and most of the animals recovered from infection after reaching a maximum of about 35% parasitaemia.

Infection and collection of sera

Rhesus monkeys were infected intravenously with 1×10^4 parasitized erythrocytes collected in 0.15 M sodium citrate from a donor with ongoing infection, Albino rats were infected intraperitoneally with 1 ± 10^6 *P, berghei* infected erythrocytes. The donor animals showing 5-10% parasitaemia during the ascending phase of infection were used. The course of infection in the infected animals was monitored once a day between 10 and 11 a.m. by thin smears of blood. The smears were fixed in methanol and stained with Jaswant Singh and Bhattacharya stain as described by

* Shepherd *et al.* (1982) have recently reported the presence of circulating immune complexes in *P. knowlesi* infected Kra and merozoite vaccinated Rhesus monkeys.

Russel (1963). The parasitized cells were counted and expressed as number of parasitized erythrocytes per 10,000 normal erythrocytes.

Blood for serum was collected from albino rats at 4 different phases of infection. Phase-I — before infection; Phase-II — during the ascending phase of infection when the animals showed 10 to 20% parasitaemia; Phase-III — during peak infection when parasitaemia was above 35% and Phase-IV — 6 days after the animals recovered from active infection. Blood for serum was collected from rhesus monkeys only during the first three phases of infection since *P. knowlesi* is highly lethal to rhesus monkeys.

General schedule for raising antisera in rabbits

Ten mg of antigen in Freund's complete adjuvant (Difco, USA) mixed at a ratio of 1 : 1 was injected in rabbits by intramuscular route on day 'O'. Second dose was given on 16th day with the same concentration of protein in complete Freund's adjuvant by the same route. Third dose was given on the 21st day with the same protein but without any adjuvant by intramuscular route. Fourth and fifth doses were given on the 22nd and 23rd days with 1/5 of the protein concentration (2 mg) used for the first dose by intravenous route without adjuvant Test bleeding was done 6-7 days after the last dose and the sera were tested by gel diffusion for the precipitin bands. Rabbits were bled on alternate days and the individual sera with good titres were pooled after testing.

Purified IgG from monkey was used at a concentration of 10 mg per dose to raise antiserum.

Anti-monkey plasma

Normal monkey plasma separated from heparinized blood was diluted in a proportion to give 10 mg/ml in normal saline and was used at 1 ml per dose per rabbit by the above mentioned schedule for raising antisera.

Anti-P. knowlesi serum

Blood from a rhesus monkey heavily infected (70-75%) with *P. knowlesi,* (mainly schizonts and trophozoites) were collected in 0.15 M sodium citrate and the cells were washed twice with chilled normal saline. The cell pellet was then diluted with equal volume of normal saline. This was quickly frozen and thawed thrice and 1 ml of this material was mixed with complete Freund's adjuvant and used for one rabbit as the first dose. The rest of the schedule was the same as mentioned above. This antiserum was absorbed with normal monkey erythrocytes overnight at 4°C by mixing equal volumes of serum and packed normal monkey erythrocytes. The serum was separated from cells after centrifugation at 850 *g* for 15 min at 4°C.

Anti-normal monkey erythrocytes

Washed normal monkey erythrocytes were diluted with equal volume of normal saline and this was frozen and thawed thrice and 1 ml of this material was used per rabbit as described earlier.

Polyethylene glycol assay (PEG assay)

The method of Haskova *et al.* (1978) was followed.

Quantitative cryoprecipitation assay

Blood was collected in a warm sterile syringe and was left for clotting at 37°C for 1-2 h in a sterile centrifuge tube. It was then centrifuged at 500 *g* for 20 min at room temperature (about 28-30°C) and 1 ml of the serum was kept at 4°C for 72 h. The cryoprecipitate formed was separated by centrifugation at 850 *g* 15 min at 4°C. The precipitate was washed twice in chilled normal saline and the final pellet was suspended in 2 ml of normal saline. The tube was then kept in a 37°C water-bath for 30 min and then centrifuged at 1300 *g* at room temperature. The supernatant was removed and its absorbance was monitored at 280 nm.

Polyethylene glycol precipitation of circulating immune complexes in serum for analysis

Essentially the procedure of Chia *et al.* (1979) was followed. To 4 ml of serum collected from a peak parasitaemic monkey, 4 ml of 8% PEG (MW-6000) in phosphate buffered saline (0.01 M pH 7.2) was added dropwise with constant stirring. After the addition was complete, the tube was left at room temperature for 1 h. It was then centrifuged at 1300 *g* for 1 h at 4°C. The resulting pellet was resuspended in 20 ml of 4% PEG and again centrifuged at the same speed. This procedure of washing with 4% PEG was repeated twice and after the last wash, the precipitate was dissolved in 2 ml of PBS 7.2 and this was used later as antigen in Immunoelectrophoresis against (i) anti-monkey IgG (ii) anti-*P. knowlesi,* (iii) anti-monkey erythrocytes, and (iv) anti-monkey plasma.

Immunoelectrophoresis

The procedure of Graber and Williams (1955) was followed using 0.05 M veronal buffer pH 8.6 in 0.8% in 0.8% agarose A (Pharmacia Fine Chemicals). Solubilized PEG precipitate was subjected to electrophoresis (7.5 mA current per microscopic slide) for 90 min and the trenches were then filled with antisera and left for diffusion overnight at room temperature in a humid chamber. Washed, dried slides were then stained with 0.5% coomassie brilliant blue.

Results

Detection of circulating immune complexes in rodent and simian malaria

The levels of the immune-complexes in the sera of rats infected with *P. berghei* as detected by quantitative cryoprecipitation and PEG assay are depicted in figure 1. Significantly high levels of circulating immune-complexes could be detected in the sera of albino rats during the ascending and peak infection and levels decreased appreciably during the post-infection period. High levels of circulating immune-complexes were detected in sera of monkeys with very high infection (parasite density more than 45%) while at the ascending phase of infection when

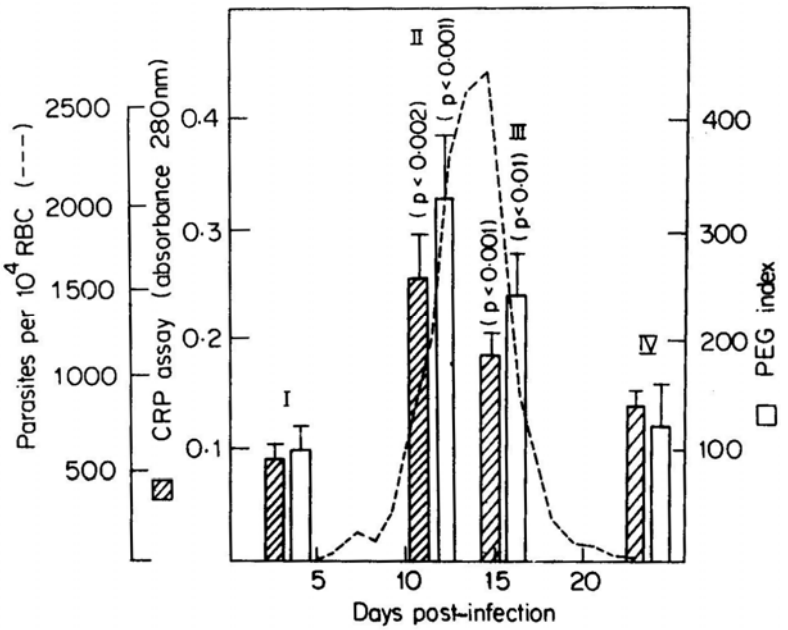

Figure 1. Content of circulating immune complexes in the sera of rats infected with *P. berghei.*

Phase-I— Control, n = 16; Phase-II – 10-20% infection, n = 18; Phase-III – more than 35 % infection, n = 10 and Phase-IV – 4-6 days post-infection, n = 20; P values are shown only when found significant i.e. P = <0.01. The course of parasitaemia in rats has also been shown (–).

parasitaemia was between 10 and 20%, there was no appreciable increase (figure 2) . The results of two tests correlated well with each other at all the stages of

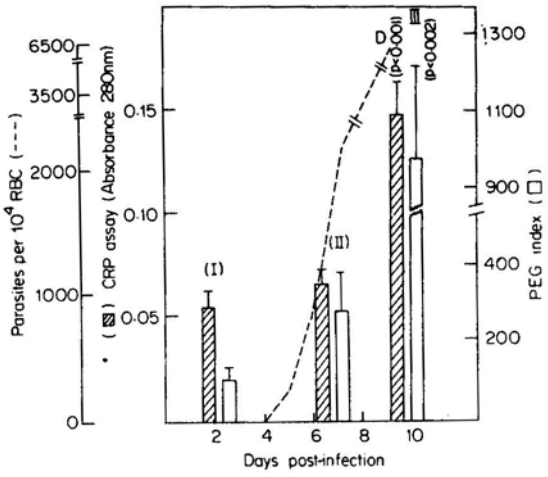

Figure 2. Content of circulating immune-complexes in the sera of rhesus monkeys infected with *P. knowlesi.* Phase-I – Control, n = 14; Phase-II –10-20% infection, n =13; Phase-Ill – more than 45 % infection, n = 14; P<0.01, The course of parasitaemia in rhesus monkeys has also been shown (–)

infection in both the rodent and the simian models, though PEG assay was found to be more sensitive than quantitative cryoprecipitation particularly in the simian model. There was a ten-fold increase in the value of PEG assay while there was only a three-fold increase by cryoprecipitation assay.

Analysis of circulating immune complexes in simian malaria

Analysis of PEG precipitate obtained from the sera of rhesus monkeys with high *P. knowlesi* infection by Immunoelectrophoresis revealed the presence of monkey IgG, four other components of monkey plasma, two components of normal monkey erythrocytes, and also antigens of *P. knowlesi* origin (figure 3a to d). Solubilised cryoprecipitate obtained from *P. knowlesi* infected sera were also subjected to immunoelectrophoresis against anti-monkey IgG and anti-*P. knowlesi* serum. Similar precipitin bands were observed as recorded with the solubilized PEG precipitate.

The soluble *P. knowlesi* antigen (s) in PEG precipitate was a heat labile component getting inactivated at 56°C in 30 min or at 100°C in 5 min (figure 3e).

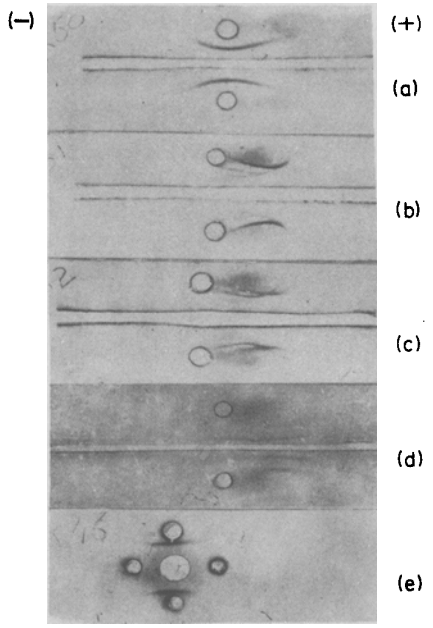

Figure 3a - d. Immunoelectrophoresis of PEG precipitate (spotted in all the wells in a, b, c and d).

After electrophoresis the trench were filled with different antisera: a= anti monkey IgG; b = anti monkey plasma; c = anti monkey erythrocytes; and d = anti *P. knowlesi.*

Figure 3e. Gel diffusion showing heat lability of *P.knowlesi* antigen in PEG precipitate — Centre well = anti-*P. knowlesi* serum. Wells above and below = untreated PEG precipitate. Well on the left = PEG precipitate heated at 56°C for 30 min. Well on the right = PEG precipitate heated at 100°C for 5 min.

Although two *P. knowlesi* antigenic components could be detected by Immuno-electrophoresis, only one component was visible by simple gel diffusion which was heat labile.

Discussion

The apparent normal values during the ascending phase of infection in monkeys could be due to the fact that in the simian system the infection proceeds in a logarithmic scale and reaches the peak within 5 or 6 days unlike the rodents where it takes at least 15 to 18 days to reach the zenith of parasitaemia. Although the principle behind the two tests, cryoprecipitate assay and PEG assay are different there was good correlation between the two at all the stages of infection in both the rodent and the simian system. The PEG assay however was relatively more sensitive for the monkey sera. It is essential to note that these two tests are considered relatively less sensitive as compared to a variety of other tests reported in the literature (Theofilopoulos and Dixon, 1979).

Since cryoprecipitate assay and PEG assay are antigen non-specific tests, it becomes essential to identify the components in the precipitate before attaching much significance to the values obtained in these tests.

The presence of monkey IgG has been identified by Immunoelectrophoresis in both cryoprecipitate and PEG precipitate obtained from *P. knowlesi* infected sera. Facer (1980) successfully identified specific malaria antibody activity from circulating immune complexes of *P.falciparum* infected sera by indirect immuno-fluorescence test. However, in a precipitation reaction like immunoelectrophresis, immunoglobulin is more readily detected as an antigen than as an antibody. Four other plasma components were also identified in PEG precipitate using rabbit anti-monkey plasma. Although the components could not identified individually, some of them could be suspected to be complement split products. The presence of IgM, IgG, IgA and complement components in immune complexes has been demonstrated in tropical splenomegaly syndrome (Ziegler, 1973) and in human malaria (Houba *et al.,* 1976).

The demonstration of the presence of soluble *P. knowlesi* antigen(s) in the PEG precipitate and cryoprecipitate by Immunoelectrophoresis is interesting. Wilson and Bartholomew (1975) had raised the possibility of circulating immune-complex associated malarial antigen to be a heat stable 'S' antigen but the malarial antigen in the PEG precipitate was shown to be heat labile, as it was inactivated in 30 min at 56°C or 5 min at 100°C.

The identification of malarial antigen in circulating immune-complexes is significant, because of the established role of such complexes in induction and effector limbs of immune response of the host against parasites (Cohen, 1976).The presence of circulating immune complexes of antigen excess have been suspected to be responsible for the chronicity encountered in many plasmodial infections (Cohen and Mitchell, 1978). Although June *et al.* (1979) showed indirect evidence for the presence of specific malarial antigen and antibodies in circulating immune

complexes in the sera of mice injected with *P. berghei,* more direct proofs were needed for further confirmation.

The demonstration of the presence of two soluble components of monkey erythrocytes in circulating immune complexes confirms the earlier observations of an autoimmune response of the host against its erythrocytes during malarial infections (reviewed by Zuckerman, 1977).

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