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Immune complexes in serum of rats during infection with *Plasmodium berghei*

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Abstract. Large amounts of immune complexes were present in the serum of infected rats early in infection when parasitemias were low. As the infection progressed and parasitemia increased and then decreased, the amounts of immune complexes in the serum also fell. This result suggests that increased efficiency of complex clearance was an important factor in determining the levels of immune complexes in the serum. In high performance liquid chromatography (HPLC), the complexes in the serum migrated as a peak with material of 350 kDa and greater in mass. They sedimented in a sucrose gradient as a band with a sedimentation coefficient of 22 *s*, which was calculated to yield a mass of approximately 1100 kDa. Immunoelectrophoresis and radial immunodiffusion showed that IgG was the major immunoglobulin in the complexes. As the IgG content of the complexes increased, the levels of complexes in the serum generally decreased. HPLC analysis of precipitated complexes suggested that they contained loosely bound albumin. Serum proteins were affected by the infection. A depletion of free immunoglobulin was observed during the initial period of immune complex formation.

Large amounts of circulating immune complexes (ICs) are a common feature of malaria. However, the clearance of these complexes and the resulting pathologic changes are not well understood. Malaria ICs collected during infection are immunosuppressive. Immune complexes, for example, collected from rats have inhibited the induction of immunity by a blood-stage vaccine (Alder et al. 1987), the in vitro antibody response to sheep red blood cells by sensitized spleen cells (Cox et al. 1983) and induction of immunity by a pertussis vaccine (Viens et al. 1974). These complexes have also inhibited in vitro phagocytosis of infected erythrocytes (Packer and Kreier 1985), and of free plasmodia (Brown and Kreier 1982; 1986). Inhibition of phagocytosis by IC is probably mediated by inhibition of Fc-receptor function (Shear 1984).

Ingestion of ICs initiates processes that activate macrophages (Pestel et al. 1981). Activated macrophages destroy ingested antigens rapidly and are thus less efficient for antigen presentation than resting macrophages (Biozzi et al. 1984). Some of the immunosuppressive effects of IC may therefore be a result of IC-mediated macrophage activation. Another method whereby ICs may affect immunity is by tying up antiparasitic antibody; both parasite antigens and antigen excess complexes may act by this mechanism. A study by Druilhe and Khusmith (1987), for example, linked immunity to malaria with a high level of antibodies that promote phagocytosis of merozoites. If antibody of the specificity needed for such phagocytosis were bound to complexes or free antigen, it would block protection. Antibody binding by immune complexes thus could shield the parasite from antibody and be a protective mechanism as stated in the smokescreen hypothesis advanced by Wilson (1974) and developed by Kreier et al. (1983).

In view of the importance of malaria ICs in the pathogenesis of malaria, we investigated the size and composition of the ICs that form and are cleared during the course of infection.

Materials and methods

Parasites. Plasmodium berghei berghei stabilates were prepared and maintained in liquid nitrogen by standard techniques

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(Trager and Jensen 1980). The frozen plasmodia were thawed as needed and infection was initiated by intraperitoneal injection (ip) into Swiss mice. After the infection was established in Swiss mice, it was passed to rats by ip injection of infected blood.

Animals. Outbred adult Sprague-Dawley (S-D) rats were used as the source of all serum samples and parasite suspensions.

Normal and immune serum. Rats were anesthetized with ether and blood was collected by cardiac puncture. Serum was separated by standard means (Alder et al. 1987). Serum samples were stored at $-20°$ C. Immune serum was collected from rats that had recovered from multiple cycles of *P. berghei* infection. These rats were inoculated ip with 2×10^8 *P. berghei*-infected erythrocytes four times at biweekly intervals. One week after the last injection, the rats were exsanguinated and the immune serum was collected and stored at $-20°$ C.

Polyethylene glycol precipitation of immune complexes (ICs). ICs were recovered from serum of adult SD rats which had been infected by intravenous injection of 10^8 infected red cells. Serum was collected at various times, ranging from 2 to 35 days postinfection, and stored at $-20°$ C. Serum from all rats bled on the same day postinfection was pooled. There were eight rats infected for each day on which serum was to be collected. The complexes were removed from serum by precipitation with polyethylene glycol 6000 (PEG, Sigma, St. Louis, MO). The pooled rat serum was mixed with a sufficient amount of 40% PEG in borate buffer (0.1 m*M* boric acid, 25 m*M* disodium tetraborate, 75 m*M* NaCl, pH 8.4) to yield a final concentration of 3.5% PEG. The mixture was incubated at 4° C overnight and the ICs were collected by centrifugation at 12800 *g* for 2 min. The pellet of complexes was washed 3 × with a 3.5% solution of PEG, then resuspended in sufficient volume of 0.85% saline to restore the original volume of the serum.

High Performance Liquid Chromatography (HPLC). Chromatography was carried out on an Altex HPLC apparatus. The buffer used contained 0.1 *M* Na_2PO_4, and 0.02 *M* NaH_2PO_4 at pH 6.8. A Bio Rad TSK 250 sizing column, with a fractionation range of 40–350 kDa, was run at $1.0 \text{ ml} \cdot \text{min}^{-1}$ and the output was continuously monitored at 280 nm. Samples consisted of 20 µl of redissolved PEG precipitate, or 20 µl of a 1:3 dilution of serum. The areas under the various eluted peaks were automatically calculated using a Hewlett-Packard integrator. Protein concentrations of the various peaks were calculated by correlating the areas of serum IgG, IgM, and albumin with standards of known protein concentration as calculated by the Folin-phenol protein assay (Lowry et al. 1951). The estimation of retention time versus molecular weight was based on the use of the same standards. The IC concentration was calculated as the material in the PEG precipitate that was >350 kDa in mass.

Sucrose-gradient ultracentrifugation. The size of the IC was estimated by ultracentrifugation (Benveniste and Bruneau 1979). The calculation of *s* values was also performed as recommended by Benveniste and Bruneau (1979). The correlation of *s* values and size of the complexes was calculated by the method of Salemme and Zubay (1983).

Immunoelectrophoresis. Immunoelectrophoresis of the samples in the sucrose gradient and the PEG precipitates was performed using standard techniques (Garvey 1977).

Radial immunodiffusion. Radial immunodiffusion was used to determine the amounts of immunoglobulin present in PEG precipitates from rat serum collected at the various times during infections (Garvey et al. 1977).

Radioimmune precipitation assay. The radioimmune precipitation (RIP) assay was used to analyze the antigens in *P. berghei* immune complexes and to examine the relative amounts of antibody and antigen in the complexes. Plasmodia were was labeled in culture by metabolic incorporation of [^{35}S]methionine (Alder and Kreier 1984). Following the in vitro labeling, the infected red blood cells were disrupted by sonication. The supernatant fluid containing the labeled antigen was collected following centrifugation at 10000 *g* for 10 min.

The RIP assay was performed using two different procedures. The first analyzed the PEG precipitates following direct reaction with labeled antigen; the second analyzed the precipitates after reaction first with hyperimmune serum and then with labeled antigen. In the first procedure, the PEG precipitates were resuspended to the original volume of the serum from which they were precipitated (1 ml) and a 50 µl aliquot was mixed with 50 µl of labeled antigen along with 50 µl of 2% DL-methionine. The mixture was incubated at room temperature for 2 h. Immune complexes and the bound labeled antigen were collected on 50 µl of protein A-coated latex beads (Sigma). In addition to the bead suspension, 150 µl of radioimmune precipitation assay (RIPA) buffer (0.05 *M* Tris, 0.15 *M* NaCL, 1% 5 Na deoxycholate, 1% Triton-X 100) was added. The mixture was then incubated at room temperature for 30 min. The latex beads to which the immune complexes were bound were pelleted by centrifugation at 12800 *g* for 2 min. The pellets were washed 5 × with 150 µl RIPA buffer per wash. Next, the complexes bound to the pellets were solubilized in a SDS-B mercaptoethanol buffer, and polypeptides were separated by a discontinuous polyacrylamide gel electrophoresis (PAGE) system (Laemmli 1973). A 4% stacking and 10% separation gel was used, and the electrophoresis was carried out at 30 mA per gel (Allen et al. 1977). The gels, fixed in 10% acetic acid overnight, were processed for autoradiography by two 30-min washes in DMSO, followed by a 3-h immersion in DMSO + 20% PPO, then by two 30-min washes in distilled water, as previously described (Alder and Kreier 1984). The gels were dried in a Bio-Rad gel drier and exposed to Kodak X-Omat x-ray film for two weeks at $-70°$ C.

The second RIP assay procedure also utilized the PEG precipitates from 1-ml serum samples collected at various times during infection, but the ICs were first converted to antibody excess form by reaction with hyperimmune serum. The pellets were each resuspended to 0.5 ml ($^1/_2$ original volume) in saline, to which 0.5 ml of serum from immune rats was added. This mixture was incubated for 2 h at room temperature. Then, 50 µl of protein-A coated latex beads were added, followed by incubation at room temperature for 30 min. The latex beads were pelleted and washed as before, then resuspended in 50 µl of labeled antigen along with 50 µl of DL-methionine and 150 µl RIPA buffer. The mixtures were incubated at room temperature for 2 h. The latex beads were again pelleted and washed five times. The immune complexes bound to the pellets were solubilized and the component polypeptides were separated by PAGE, followed by autoradiography as described above.

Results

Parasitemia in the course of infection

Serum was collected from rats during infection with *Plasmodium berghei.* Table 1 shows the par-

Table 1. Parasitemia in *P. berghei* infected rats on days serum used in this study was collected

Day of collection	Parasitemia level
0	0
2	0.5
5	3.2
9	11.7
14	18.9
17	21.5
26	0
35	0

asitemias at the times of collection. The pattern of infection and recovery is typical of malaria infection in young S-D rats. The serum samples collected were used in experiments to characterize the circulating immune complexes.

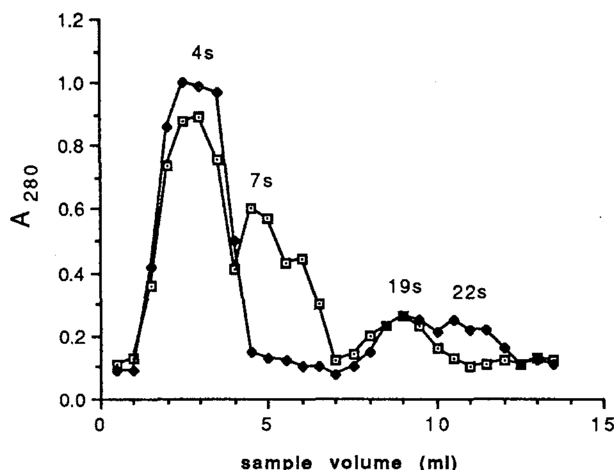

Fig. 1. Fractionation by sucrose-gradient ultracentrifugation of normal serum and of serum taken 5 days postinfection. Note the reduced protein level in the 7 *s* region and the increased amount of protein in the broad 22 *s* region in the postinfection serum. —□— normal serum; —◆— 5 day post-infection serum

Characterization of the immune complexes by sucrose gradient ultracentrifugation

Centrifugation through a sucrose gradient was used to determine the size of the immune complexes. A representative fractionation pattern of normal rat serum and serum collected 5 days after challenge (parasitemia = 3.2%) is shown in Fig. 1. Sera from infected individuals contained material that sedimented at 22 *s*. No material in normal rat serum sedimented at 22 *s*. Serum from infected rats had a reduced amount of material that sedimented at 7 *s*. The 22 *s* fraction from infected rat sera contained IgG but not IgM, as demonstrated by immunoelectrophoresis (data not shown). No IgG was found in any fraction of normal serum that sedimented at a value >7 *s*.

The mass of the complexes was estimated by two different techniques. The first technique was based on the sedimentation constant of the immune complexes (22 *s*) and of 995 kDa IgM (19 *s*), the molecular mass of the complexes was estimated to be 1240 kDa using the formula:

$$^{s}\text{unknown}/^{s}\text{known} : [^{\text{kDa}}\text{unknown}/^{\text{kDa}}\text{known}]^{2/3}$$

introduced by Salemme and Zubay (1983). The second technique utilized a graph in which were plotted *s* values vs molecular mass of known substances. The substances used to construct the graph were: cytochrome *c*, 13.4 kDa, 1.17 *s*; myoglobin, 16.9 kDa, 2.04 *s*; chymotrypsinogen, 23.2 kDa, 2.54 *s*; B-lactoglobulin, 37.1 kDa, 2.9 *s*; serum albumin, 68.5 kDa, 4.6 *s*; hemoglobulin, 64.5 kDa, 4.5 *s*; catalase, 247.5 kDa, 11.3 *s*; urease, 482 kDa, 18.6 *s*; IgM, 1000 kDa, 19 *s*; and tobacco mosaic virus, 40590 kDa, 198 *s* (graph not shown). Interpolation of the 22 *s* value of immune complexes into this graph yielded a molecular mass of 1030 kDa for the immune complexes.

Analysis by HPLC of serum samples collected during the course of infection

Serum samples collected during the course of infection were fractionated by HPLC to analyze changes in components relating to the immune complex content of the serum. Normal rat serum resolved into four major fractions which corresponded to IgM and other high molecular mass compounds (>350 kDa), IgG (160 kDa), albumin (65 kDa) and small lipoproteins (40 kDa) (Fig. 2A). In the serum samples collected during the period of developing parasitemia, the areas of the peaks that corresponded to free IgG decreased in size, while the peaks that corresponded to components >350 kDa increased (Fig. 2B, C). The peak that corresponded to free IgG reached its minimal value on day 5, when the parasitemia was 3.2%. At this time, the maximal concentration of high molecular weight material was present (Fig. 2C). As parasitemia increased, the free IgG peak remained low, while the concentration of high molecular mass components decreased (Fig. 2D). As parasitemia was cleared, the amounts of IgG and of high molecular mass material in the serum gradually returned to preinfection levels (Fig. 2E, F).

Fig. 2A–F. HPLC fractionation profiles of normal rat serum and of serum collected from rats on different postinfection days (p.i.). **A** Normal. **B** Day 2 p.i. **C** Day 5 p.i. **D** Day 17 p.i. **E** Day 26 p.i. **F** Day 35 p.i. *m*, molecular mass. See text for explanation

Analysis of immune complexes precipitated from serum samples collected during the course of infection

Precipitation with PEG at a final concentration of 3.5% was used to separate IC from the majority of other serum components, including free IgG and IgM. The precipitates were then analyzed by HPLC; a representative pattern is shown in Fig. 3. The precipitate from serum collected 5 days after infection yielded two peaks which corresponded to the putative complexes >350 kDa and 60–65 kDa components which is in the size range of albumin. The precipitate prepared from normal rat serum under the same conditions contained only a trace of material in the 60–65 kDa range, and no IgG or IgM. Precipitates from serum collected at other times during infection yielded patterns similar to that characteristic of the 5 day sera

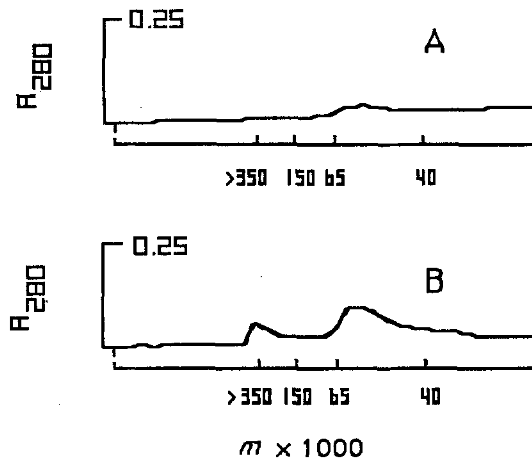

Fig. 3A, B. HPLC fractionation profiles of the 3.5% PEG precipitates from S-D rat sera. **A** Normal; slight peak in the 60 kDa region. **B** Day 5 p.i.; distinct peaks in the >350 kDa and 60 kDa regions

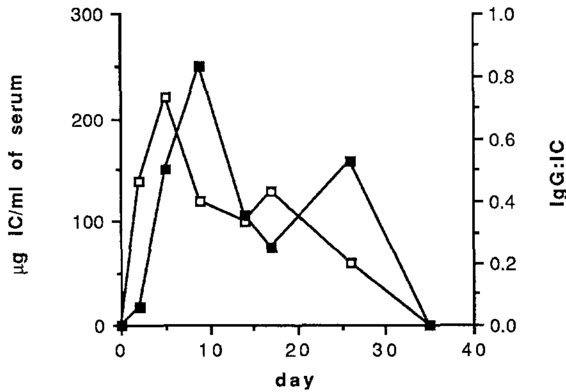

Fig. 4. Graph showing amount of IgG (determined by immunodiffusion) and of total IC protein (determined by absorption at 280 nm) in infected rat serum during infection. Note the rise in the level of the complexes in early infection (days 5–9 p.i.) followed by a decrease; all complexes were cleared by day 35 p.i. –□– IC; –■– IgG:IC

(data not shown). No peaks were found in the mass range of free IgG in any precipitate.

Estimation of the amounts of circulating ICs during infection

The total amounts of circulating ICs in the serum samples were calculated by the absorbance at 280 nm of the fraction of the PEG precipitate with mass >350 kDa. The amount of IgG in the complexes was calculated by radial immunodiffusion of the PEG precipitates. By immunoelectrophoresis, the complexes were shown to contain IgG (not IgM), presumably bound in IC. Immunoelectrophoretic analysis also showed that the PEG precipitate from normal serum contained neither IgG nor IgM (data not shown). The precipitation procedure thus isolates IC and not free IgG of IgM.

The amounts of ICs in serum increased rapidly in the initial stages of infection and a peak concentration of 220 µg/ml was reached on day 5 (Fig. 4). The concentration of complexes then decreased in an irregular fashion for the remainder of the course of infection. No complexes were detected on day 35.

The proportion of IgG in the complexes was initially quite low. At day 2 there was only 1/10 as much IgG as plasmodium protein in the complexes while on day 5, they contained about 3/5 as much IgG as parasite protein. As parasitemia increased and resolved, there was generally more plasmodium protein than IgG in the complexes. The total amount of complexed material in the serum was shown by the analysis of the PEG precipitates as by the HPLC analysis (Fig. 2) to increase rapidly, reaching a peak early in the course of the infection, and then, slowly to decrease. The decrease started and continued during rising parasitemia.

Determination of plasmodium antigens in the ICs by immunoprecipitation

ICs isolated from sera collected after recovery (day 26) bound more free, radiolabeled plasmodium antigen than did complexes isolated from sera during parasitemia (Fig. 5, lanes 8 and 10). The complexes isolated from day-26 sera also

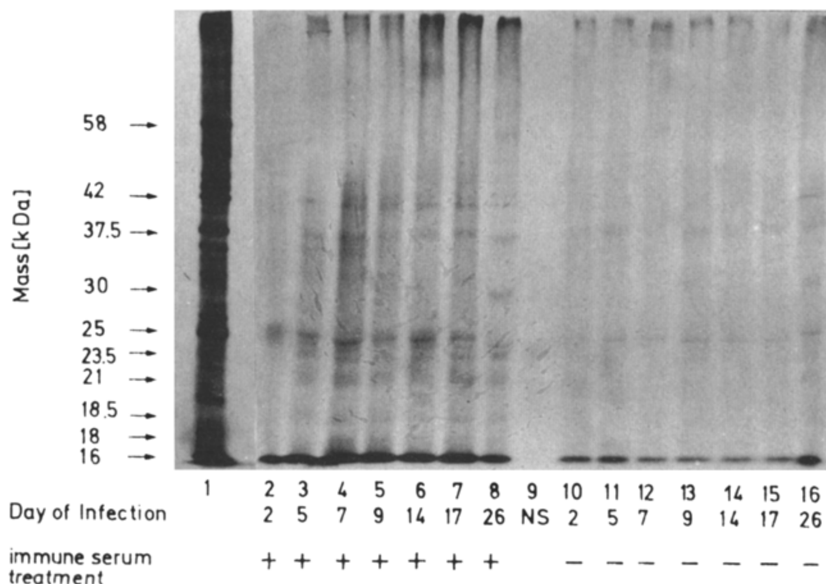

Fig. 5. Autoradiograph showing the results of RIP analysis of ICs collected in the course of infection. In general, ICs pretreated with immune serum bound more labeled antigen (*lanes 2–7*) than untreated ICs (*lanes 10–15*). However, the ICs collected on day 26 p.i. bound similar amounts of antigen when pretreated with immune serum and when directly exposed to labeled antigen irrespective of whether they were or were not pretreated with immune serum (*lanes 8 and 16*). Normal rat serum did not precipitate enough labeled antigen to form any bands

bound several radiolabeled peptides (58 kDa, 30 kDa) of mass not bound by complexes recovered from serum samples collected in earlier infection stages. The ICs incubated with immune serum before exposure to radiolabeled malaria antigen bound larger amounts of labeled peptide, but in general, did not react with different peptides than did immune complexes reacted directly with labeled antigen. The exceptions were ICs isolated from serum collected after recovery (day 26) which bound about the same amount of radiolabeled antigen before and after incubation with immune serum (Fig. 5, lanes 8 and 16).

Discussion

As infection progressed, a variety of interrelated changes occurred in the blood of the infected rats. These included: plasmodium growth, release of parasite products, and the induction of various host responses. There was a lack of correlation between parasitemias and the amounts of ICs detected. As parasitemia increased between days 5 and 17, the concentration of ICs in the serum actually decreased. This indicated that as infection progressed, the rate of removal of parasite debris increased more rapidly than did its rate of formation.

It has been reported that complexes containing bovine serum albumin as antigen had a half life of 6 min in the circulations of immunized animals, compared to 24 h in the circulations of normal controls (Day et al. 1980). The rate of IC clearance is thus determined in part by the state of the host's immunity. As clearance of malaria IC in rats accelerated early in the disease, it is probable that immunity also began to develop early. This immunity eventually caused the elimination of the parasites from the blood. Prior to the elimination, there was a decrease in the amounts of circulating ICs and the increase in the amounts of IgG in the complexes.

It is probable that antibody production and macrophage activation both contribute to clearance of parasite debris. In this study, we did not evaluate macrophage function but there is evidence to suggest its importance in malaria (Chow and Kreier 1972; Brooks and Kreier 1978; Shear 1984; Brown and Kreier 1986; Druilhe and Khusmith 1987). Our determination of antibody content in ICs, however, indirectly suggests that induction of antibody production was important in complex clearance, since in general as infection progressed, the proportion of immunoglobulin in the complexes increased and the complexes richer in IgG were formed during the period of IC decrease.

We used radial immunodiffusion to determine the amounts of immunoglobulin in the complexes. This procedure in which anti-rat immunoglobulin and ICs were allowed to react in a 2% agarose matrix may have worked because the complexes disassociated as the components diffused away or because the agarose has a pore size sufficiently large to allow free diffusion of even the largest complexes.

Our data also revealed something about the size of the immune complexes present in rats with plasmodium infection. The HPLC fractionation patterns of whole serum showed that the complexes had a mass >350 kDa. The sucrose-gradient centrifugation further indicated that the complexes sedimented at 22 *s* and thus had a molecular mass of about 1100 kDa, indicating they were only slightly larger than IgM which sediments at 19 *s*. While IgM certainly made up a fraction of the material >350 kDa in the whole serum, IEP analysis detected only IgG and not IgM in the ICs isolated by PEG precipitation or by ultracentrifugation. Since the IgG subclasses have a mass in the range of 150 kDa and soluble plasmodium antigens may have a mass in the 50–100 kDa range, the complexes probably contain only a total of 8–10 molecules.

The ICs were precipitated from the serum by polyethylene glycol. This procedure yielded complexes free of unbound antibody. HPLC analysis of the complexes precipitated from serum samples collected during infection consistently yielded two peaks. One peak was 350 kDa and greater, which represents the ICs, and a second peak was 60–65 kDa which is in the size range of albumin. Therefore, the IC seem to be composed largely of IgG, parasite components, and some albumin. HPLC analysis showed that the albumin content of the whole serum samples remained relatively unchanged during infection. Therefore, the amounts of albumin complexed were not sufficient to affect their total levels in the serum, but did constitute a detectable component of the immune complexes.

HPLC analysis of the PEG precipitates resolved the albumin as a component distinct from the high molecular mass immune complexes. Therefore, while albumin seems to be associated with the ICs, probably through binding to the parasite antigen, the strength of the albumin-immune complex association is apparently sufficiently weaker than are the antigen-antibody bonds to permit dissociation during analysis. Current research on this topic indicates that some parasite components do bind albumin (Kreier et al. 1987).

The levels of circulating ICs did not directly correlate with parasitemia. The peak in immune complex concentration (200 μg/ml) occurred early in infection. During the period in which parasitemia increased most rapidly (days 9–17), the immune-complex level stabilized for a period at 100 μg/ml. In mice lethally infected with *P. chabaudi,* the concentration of circulating complexes appeared to remain constant at a high level as the parasitemia rapidly increased during the last days of infection (Cox et al. 1984). Thus, the concentration of ICs appears to stabilize at a high level in lethally infected mice and for a period at a lower level in nonlethally infected rats. This leveling off in concentration of circulating ICs in both lethally and nonlethally infected animals suggests that the clearance increases as the disease progresses in both types of infections.

A radioimmune precipitation assay was used to determine the antigens present in the complexes. In this procedure, free antibody and radiolabeled antigen were reacted with the complexes. The results obtained so far are not completely satisfactory, but the procedure has promise. Since protein A was used to recover the complexes, it is possible, for example, that free antibody which attaches directly to the protein A was at least partially responsible for binding of the radiolabeled antigen observed. However, as complexed IgG has a much higher affinity for protein A than free IgG (Kessler 1975), it is probable that binding of IgG by protein A had only a minor effect on the results. The similarity of the labeled polypeptides bound by the complexes following the addition of hyperimmune serum to those bound directly by the complexes also suggests that the procedure detected antigens in the complexes. The most notable conclusion suggested by the radioimmune precipitation assay results is that a rather wide range of parasite components are present in the complexes and, therefore, many different plasmodium components are released into the plasma during infection.

The course of the changes in immunoglobulin levels found in the sera was unexpected in that the free IgG decreased to quite low levels before it gradually recovered as infection progressed. It is impossible to account for a decrease in immunoglobulin levels of the magnitude observed by postulating complexing with parasite antigens alone, since even in the most stimulated system only a fraction of the immunoglobulin has any given specificity. Depression of production probably did not occur in the course of rising parasitemia, since the amounts of IgG in the complexes increased as the disease progressed. A more rapid rate of utilization than production is thus the most likely cause of the decrease in free IgG observed. Although this study was not concerned with autoimmune and nonspecific mechanisms of immunoglobulin utilization, it is possible that such mechanisms contributed to the initial decrease in free IgG.

There is no necessary relationship between antibody titers to a particular antigen and total IgG levels. While it is true that during nonlethal infections, specific antibody titers generally increase during recovery, no conclusions should be drawn about antiplasmodium titers on the basis of the observed changes in IgG levels. In fact, a strong antibody-dependent immunity develops slowly in the S-D rats used in this study. It usually requires at least three cycles of infection and recovery to produce sufficient protective antibody in the rat sera so that a strong immunity can be induced by passive transfer. Therefore, the depletion of free IgG observed in the rats during an initial infection yields no information about immunity after recovery.

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