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Immunopathological aspects of *Plasmodium berghei* infection in five strains of mice

I. IMMUNE COMPLEXES AND OTHER SEROLOGICAL FEATURES DURING THE INFECTION

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SUMMARY

The development of circulating immune complexes was studied in mice of the BALB/c, A/J, OF_1, CBA and C57Bl strains infected with *P. berghei*. Complexes were evaluated in relation to levels of parasitaemia, soluble antigen, specific antibody and C3. Susceptibility to infection was greatest in BALB/c, A/J and $OF/_1$ mice. The maximum parasitaemia was 30% in CBA and 70% in all other strains. Levels of soluble antigen paralleled those of parasitaemia. Specific antibody was detected in all strains, but the titre continued to rise throughout the infection only in CBA mice. Circulating immune complexes occurred in mice of all strains from day 6; the level fell after day 9 in C57Bl whereas it was maintained in CBA mice. The development of immune complexes was associated with marked depression of C3 levels in all except CBA mice, in which a transient reduction was followed by recovery. Partial characterization of the complexes showed that IgM-containing complexes appeared earliest and reached highest levels in BALB/c mice while in CBA mice, IgM complexes were found in lesser amounts and the level fell in late infection. IgG complexes rose throughout infection in CBA and fell in later stages in BALB/c and C57Bl mice. In nude BALB/c mice, immune complexes were usually not detectable and only low levels of antibody of IgM class were produced. Differences in mortality pattern could not be related to any single serological factor.

INTRODUCTION

It has been shown that the outcome of malaria infection depends on factors related to both the host and the parasite (Greenberg & Kendrick, 1957a). In mice infected with *P. berghei*, a range of susceptibility is seen in different strains. A more or less rapidly fatal issue is generally observed (Eling, Van Zon & Jerusalem, 1977) but occasional spontaneous recovery has been described in NMRI mice (Kretschmar, 1962). The basis of the variation in susceptibility is not fully understood. However, it does not appear to depend on strain adaptation of the parasite (Greenberg & Kendrick, 1957b). Little correlation was found between parasitaemia 6 days after infection and survival in mice of different strains and studies on the F_1 and backcross generations indicated that several genes influence the course of infection (Greenberg & Kendrick, 1958). It has been suggested that strain differences in the immunopathological effects of infection may be related to differences in the host

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immune response, leading to differences in the cellular immune response, to the amount of autoantibody production and to other physiological factors such as the concentration of ATP in the red blood cells (Brewer & Powell, 1965).

We have studied immunopathological aspects of *P. berghei* infection in one strain of mice and shown that circulating immune complexes and profound alteration of the complement system occur and that these changes progress during the course of the infection (June *et al.*, 1979a). In the present study, we have investigated serological and immunopathological features of malaria infection in five strains of mice infected with the same isolate of *P. berghei*, to determine whether differences can be shown in the serological abnormalities and whether these can be related to differences in susceptibility to infection and to the pathogenesis of tissue lesions. The pathological findings are reported separately (Mackey *et al.*, 1980).

MATERIALS AND METHODS

Mice. Seven-week-old female outbred OF_1 and inbred A/J, CBA, C57Bl and BALB/c mice were purchased from IFFA CREDO (Centre de Recherche et d'Elevage des Oncins, St Germain sur l'Arbresle, France). Congenitally athymic BALB/c nu/nu mice were purchased from Gl. Bomholtgard Ltd (Ry, Denmark).

Parasite. Plasmodium berghei strain ANKA was maintained by weekly blood passage in OF_1 mice.

Malaria infection. Sixty mice of each strain, and 10 BALB/c nu/nu mice were injected intraperitoneally with 5×10^5 parasitized red blood cells (pRBC). The pRBC used to infect mice of different genetic backgrounds were obtained from an infected mouse of the same background, which had in turn been infected by parasites obtained from infected OF_1 mice. The BALB/c nude mice were infected with blood obtained from normal BALB/c mice. At 3-day intervals beginning 3 days after infection, five mice from each group were killed by exsanguination from the retro-orbital plexus. The group of BALB/c nu/nu mice were killed 9 days after infection. The blood was allowed to clot for 1 hr at room temperature and 1 hr at 4°C; the serum was recovered by centrifugation at 1,500 ***g*** for 10 min and immediately stored at −70°C in small aliquots.

Parasitaemia was determined from blood smears in all mice by tail bleeding immediately prior to killing. Mortality was recorded each day. The percentage of mice dying was calculated after first excluding those mice which had been killed. The kidneys of two different mice from each strain were snap-frozen and stored in liquid nitrogen within 10 min of death.

Immunofluorescent antibody titre. The indirect fluorescent antibody technique was used to determine the serum levels of antibodies to malaria using antigen slides prepared from pRBC taken from each strain during the reticulocytic phase of infection (O'Neill & Johnson, 1970; June *et al.*, 1979a).

C1q-binding assay (C1q-BA). A microassay adaptation of the C1q-binding test of Zubler *et al.* (1976) was used for the detection of soluble immune complexes which allowed increased sensitivity in mouse serum and a reduction of required sample volume (June *et al.*, 1979b). In brief, 10-μl samples were mixed with 20 μl of 0·3 M EDTA at pH 8·3 in removable flat-bottomed polystyrene wells (Cooke Laboratory Products, Alexandria, Virginia). After incubation at 37°C for 30 min, 10 μl of ^{125}I-C1q and 200 μl of 3% polyethylene glycol in borate buffer, pH 8·3, were added. Following an incubation at 4°C for 3 hr, the wells were centrifuged at 1,000 ***g*** for 30 min at 4°C. To facilitate the drainage of the wells on inversion, one drop of 30% Tween 20 in normal saline was added to each well. The wells were immediately inverted and the residual radioactivity was determined. The results were expressed as the percentage of radioactivity precipitated compared to wells in which 200 μl of 20% trichloroacetic acid were added as the precipitant. This value represented between 92 and 99% of the total radioactivity input.

Conglutinin-binding solid-phase radioimmunoasssay (KgB-SP). The C3-dependent KgB-SP, as described by Casali *et al.* (1977), was performed with two modifications. First, monospecific rabbit antisera to mouse IgM and IgG were used in order to detect immune complexes containing IgM or IgG, and secondly, the test was adapted to a microassay system (June *et al.*, 1979a).

Antisera were prepared by immunizing rabbits with mouse myeloma proteins IgM or IgG (Bionetics, Kensington, Maryland). The rabbit antisera were then purified by affinity chromatography using columns of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) coupled with either mouse IgM or mouse IgG (Axen, Porath & Ernbach, 1967). The purified antibodies were eluted with 2·5 M KSCN dialysed against PBS. The two different preparations did not cross-react by immunoelectrophoresis or double gel diffusion. They were radiolabelled with ^{125}I (Radiochemical Centre, Amersham. England) by the chloramine T method (McConahey & Dixon, 1966).

The test was performed in polystyrene wells (Cooke) coated with purified bovine conglutinin by addition of 100 μl of conglutinin at a concentration of 5 μg/ml. The samples to be tested were diluted 1:40 in veronal-buffered saline containing calcium, magnesium and 0·025% Tween 20 (VBS–Tween) and then 200 μl were added to the wells. After a 2-hr incubation at room temperature, the wells were washed three times with VBS–Tween. Then, 100 μl of either ^{125}I-anti-mouse IgM or ^{125}I-anti-mouse IgG, at a concentration of 2 μg/ml, were added to the wells, and the wells were incubated for 4 hr at room temperature. The wells were then washed three times with VBS–Tween and counted for residual radioactivity. Serial dilutions of complement-reacted aggregated mouse IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) served as positive controls for the detection of IgG- containing CIC.

Samples were tested in duplicate and without prior thawing for the C1q-BA and the KgB-SP. Those samples with values greater than 2 standard deviations above the mean value obtained using normal mouse sera were considered positive.

Immunochemical techniques. Serum C3 levels were determined by single radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) using goat anti-mouse C3 (Cappel Laboratories, Downington, Pennsylvania). Results were expressed as the mean of duplicate determinations compared to a pool of sera from normal mice of each strain.

Malarial antigens were detected using counterimmunoelectrophoresis (June *et al.*, 1979a). Mouse hyperimmune serum raised in OF_1 mice served as the antibody source. Results for positive sera were expressed as the number of antigen precipitin lines detected.

Statistical analysis. Spearman's rank order analysis (with all values in the normal range being assigned the same rank) and the Student *t*-test (corrected for populations of unequal variance) were performed.

RESULTS

Course of the infection

After infection with 5×10^5 *P. berghei*-infected red cells, all mouse strains had a prepatent period of 3 to 6 days (Fig. 1). During the second and third weeks of infection, levels of parasitaemia were similar for all strains except the CBA mice. Eighteen days after infection, parasitaemia was 25 ± 30·8% in CBA, 63 ± 14·1% in A/J, 55 ± 10·8% in BALB/c, 46 ± 11·9% in C57Bl and 58 ± 13·0% in OF_1 mice.

As shown in Fig. 2, the first death occurred approximately 9 days after infection in all mouse strains except CBA, in which there were no deaths until day 15. CBA mice had both lowest mortality and the latest onset of death.

Detection of soluble P. berghei *antigens*

Soluble malarial antigens were detected in serum using counterimmunoelectrophoresis. Precipitin lines appeared first on day 6 after infection in five of five BALB/c and C57Bl mice, on day 9 in five of five A/J and OF_1 mice, and four of five CBA mice. Thereafter, antigens were detected in all mice in all groups except CBA. In general, more precipitin lines appeared as infection progressed and parasitaemia rose. Up to four precipitin lines were detected in advanced infection. In CBA mice, which developed varying levels of parasitaemia, few, or in some cases no, precipitin lines were detected after day 15.

Normal sera from all mouse strains were negative when tested by counterimmunoelectrophoresis against the hyperimmune anti-malarial serum raised in OF_1 mice.

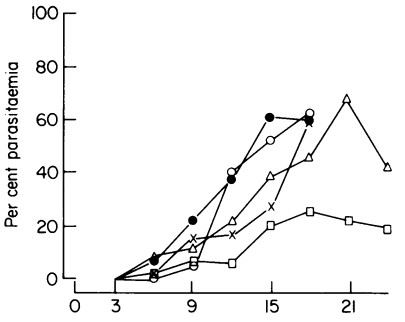

Fig. 1

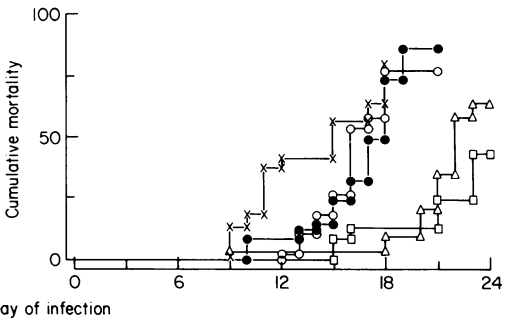

Fig. 2

Fig. 1. Parasitaemia during *P. berghei* infection in five strains of mice: each point represents the mean parasitaemia in five mice from each strain killed at 3-day intervals beginning 3 days after infection. The percentage of infected RBC was determined by counting 500 RBC in thin blood smears. (o——o) A/J, (●——●) BALB/c, (▵——▵) C57Bl, (□——□) CBA, (× —— ×) OF_1.

Fig. 2. The cumulative mortality in five strains of mice infected with *P. berghei*: at 3-day intervals beginning on day 3 after infection five mice from each group were killed. In the remaining mice the mortality was recorded daily. (o——o) A/J, (●——●) BALB/c, (▵——▵) C57Bl, (□——□) CBA, (× —— ×) OF_1.

Serum-specific antibody levels

Malarial antibody was first detected 6 to 9 days after infection in all strains of mice (Fig. 3). The titres rose rapidly and there was no difference between the strains during the first 2 weeks of infection. In the third week the titre continued to rise in CBA mice whereas the level was constant or slightly reduced in C57Bl mice. The titres of control sera did not exceed two.

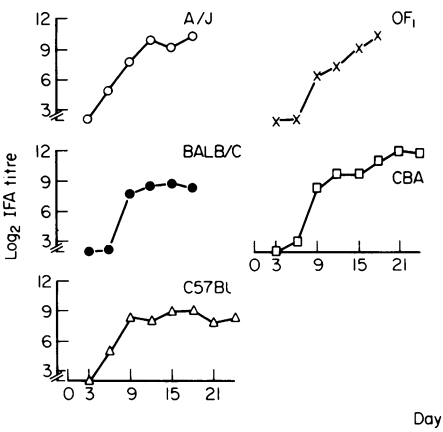

Fig. 3

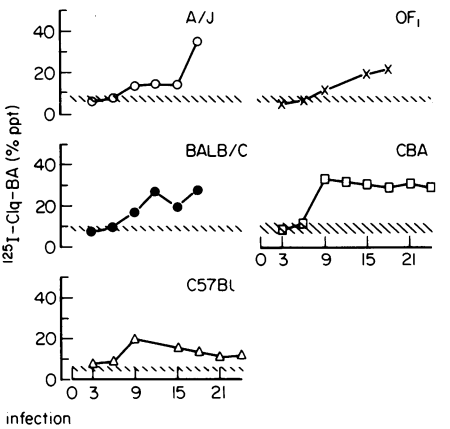

Fig. 4

Fig. 3. Serum immunofluorescent antibody (IFA) titre in five strains of mice infected with *P. berghei*: each point represents the arithmetic mean titre from assays on each of five mice killed at 3-day intervals. The results are expressed as the $\log_2$ of the reciprocal titre.

Fig. 4. Circulating immune complex levels in five strains of mice infected with *P. berghei* as detected by the C1q-binding assay: each point represents the mean level of individual assays of five mice killed at 3-day intervals. The shaded area indicates the mean ± s.d. of eight normal mice from each strain.

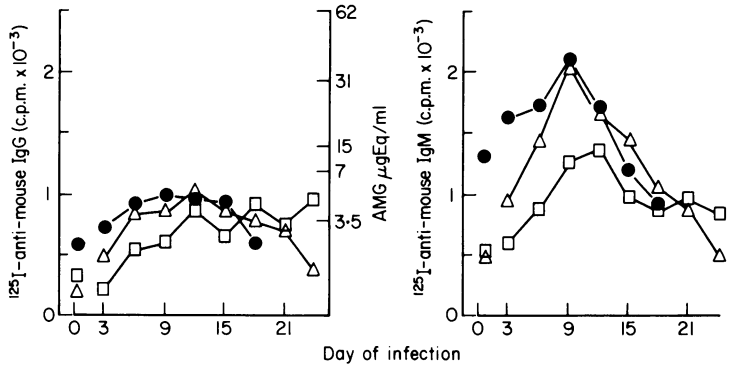

Fig. 5. IgG- or IgM-containing circulating immune complexes detected in BALB/c (●——●), C57Bl (▵——▵) or CBA (□——□) mice infected with *P. berghei*: the immune complexes were quantitated with the conglutinin-binding solid -phase assay using either ^{125}I-anti-mouse IgG or ^{125}I-anti-mouse IgM. Each point represents the mean of individual assays on five mice killed at 3-day intervals. The individual symbols (day 0) represent the mean binding of normal mice from each strain; these values were 1,229 ± 276 c.p.m. for BALB/c, 526 ± 126 c.p.m. for CBA and 482 ± 194 c.p.m. for C57Bl mice. Also shown on the left is the equivalent binding of complement-reacted aggregated mouse IgG (AMG).

Circulating immune complexes

Circulating immune complexes (CIC) were first detected using the C1q-binding assay (Fig. 4). Increased levels of CIC appeared on day 9 of infection in all mouse strains except for C57Bl mice in which slightly increased CIC levels ($P < 0{\cdot}01$) were already detected 3 days after infection. Increased levels of C1q-binding material remained present in all strains until the end of the experiment.

The conglutinin-binding solid-phase assay (KgB-SP) was used to characterize partially the immune complexes by quantitating IgM- or IgG-containing circulating immune complexes in the BALB/c, C57Bl and CBA groups of mice (Fig. 5). Sera from eight normal mice of each strain were also tested. Significant levels of IgM-containing CIC were first detected for BALB/c ($P < 0{\cdot}01$; Student's *t*-test *vs* controls) and C57Bl ($P < 0{\cdot}0001$) mice on day 6, and on day 9 for CBA mice

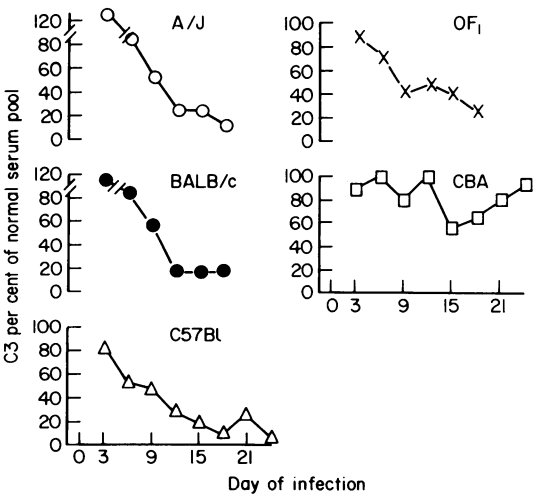

Fig. 6. Serum C3 levels as measured by radial immunodiffusion in five strains of mice infected with *P. berghei*: each point represents the mean level from individual assays on five mice killed at 3-day intervals. The mean ± s.d. C3 level in eight normal mice of each strain was 111·5 ± 26% for A/J, 100·6 ± 16% for BALB/c, 103 ± 19·5% for CBA, 105 ± 8·4% for C57Bl and 95·4 ± 14·8% for OF_1 mice.

Table 1. Relation of CIC levels to C3 consumption in *P. berghei* infection of mice

Mouse strain	Correlation C1q-BA and C3*	
	r	*P*
A/J	−0·78	< 0·0001
BALB/c	−0·82	< 0·0001
OF_1	−0·65	< 0·001
CBA	−0·35	< 0·02
C57Bl	−0·18	> 0·4

* Spearman's rank order analysis, $n = 29$ to 40 for each group.

($P < 0·02$). Peak binding was observed on days 9 or 12 and levels of IgM-CIC had either returned to normal by the end of the experiment in BALB/c and C57Bl mice or to only slightly elevated levels in CBA mice.

On the other hand, when ^{125}I-anti-mouse IgG was used to detect IgG-containing CIC, significant levels of binding were not found until day 9 for BALB/c ($P < 0·0001$) and CBA ($P < 0·0001$) mice, or until day 12 for C57Bl mice ($P < 0·0001$). Peak IgG-containing CIC levels were found later in the infection than were the peak IgM-containing CIC levels.

Serum C3 levels

All strains of mice showed a similar pattern of serum C3 levels, again except for the CBA mice (Fig. 6). In A/J, BALB/c and OF_1 mice, C3 levels were normal or increased on day 3 of infection, but they then progressively decreased throughout the experiment. C57Bl mice had slightly decreased C3 levels even on day 3 of infection (83·2 ± 7·7%; $P < 0·01$), which later became profoundly depressed. Serum C3 levels were either not detectable or less than 10% of normal in all strains during the third

Table 2. *P. berghei* infection in nude mice*

Serum	Parasitaemia (%)	C1q-BA (% ppt)	Malarial antigens†	C3 (%)	IFA titre IgM	IFA titre IgG
1	18	6·8	++	90·9	1:40	Neg.
2	10·5	6·0	++	125·6	1:160	Neg.
3	19	8·1	++	98·7	1:40	Neg.
4	17·5	13·2	++	50·6	1:80	1:10
5	28·5	6	+++	90·9	n.d.	Neg.
6	11·5	7·6	++	108·8	1:20	1:10
Mean ± s.d.	17·5±6·5					

* BALB/c (nu/nu) infected with 5×10^5 pRBC and examined on day 9. C1q-BA for BALB/c non-infected controls, 8·6 ± 0·7%. C3 for BALB/c non-infected controls, 100 ± 15·9%.

† Number of antigens detected in serum by counterimmunoelectrophoresis.

week of infection except for CBA mice. CBA mice had normal levels of C3 until the third week of infection when there was a decrease followed by a gradual recovery of serum C3 levels.

As measured by Spearman's rank analysis, there was a high correlation between serum C3 levels and C1q-binding activity in A/J and OF_1 mice, lower correlation in CBA mice, and no correlation in C57Bl mice (Table 1).

P. berghei *infection of BALB/c nude mice*

The mean level of parasitaemia in BALB/c nu/nu mice killed 9 days after infection with *P. berghei* was 17·5 ± 6·5% (Table 2), which was slightly less than normal BALB/c mice (23·2 ± 5·9%) killed on the same day of infection. Low levels of specific antibody were detected and these were found to be antibodies of the IgM class. Two or three malarial antigens were detected in the serum of both BALB/c euthymic and athymic mice, and all except one BALB/c nu/nu mouse had normal serum levels of C3. The C3 level in BALB/c euthymic mice was 56·9 ± 8·8% on day 9. In the nude mice, no CIC could be detected by the conglutinin-binding solid-phase assay using either ^{125}I-anti-mouse IgM or IgG. Low levels of CIC were found in only one of six BALB/c nu/nu mice by the C1q-binding assay (Table 2). This was the same mouse that also had a decreased C3 level. When euthymic BALB/c mice were tested 9 days after infection with *P. berghei*, four of five mice had significant levels of CIC detected by the C1q-BA (16·7 ± 12·4%, mean ± s.d.) and five of five mice had IgM- and IgG-containing CIC detected by the KgB-SP.

DISCUSSION

This experiment shows significant strain differences in mortality pattern and immunopathology in mice infected with *P. berghei*. Our results confirm earlier observations that *P. berghei* generally produces a lethal infection in mice but that mice of the A strain are particularly susceptible while C57Bl mice are relatively resistant and BALB/c mice show intermediate susceptibility (Greenberg & Kendrick, 1957a). A biphasic mortality pattern, with a first peak about 1 week after infection, which has been noted previously (Nadel *et al.*, 1955), was most apparent in BALB/c and C57Bl mice in this experiment.

Mortality was not related solely to levels of parasitaemia, since although CBA mice showed relatively long survival and also had lowest levels of parasitaemia, C57Bl mice survived as long as CBA mice but developed parasitaemia equal to that found in other strains with high mortality and acute deaths. Furthermore, early deaths occurred in A/J, OF_1 and BALB/c mice when parasitaemia was less than 20%, indicating that death was not directly related to the degree of parasitaemia and/or anaemia. Pathological examination showed no evidence of intercurrent viral or bacterial infection. It has been suggested that there is a direct relationship between the degree of NK cell response and resistance to haemoprotozoan infections in mice and that NK cells may be responsible for the destruction of parasitized RBC and consequent reduction of parasitaemia levels (Eugui & Allison, 1979). Our results are not entirely in accord with this hypothesis, since CBA and C57Bl mice showed equal resistance but widely differing levels of parasitaemia.

Soluble circulating antigen, detected in serum by countercurrent immunoelectrophoresis as described by Seitz (1975) was demonstrable first in BALB/c and C57Bl mice, which were also the strains in which the infection first became patent; in CBA mice, both lower antigen and parasite levels were found. The level of soluble antigen therefore correlated with that of parasitaemia.

A marked antibody response occurred in all strains, with identical profiles in the first 2 weeks of infection. In CBA and C57Bl mice, there was a notable difference in the third week, when the titre continued to rise in CBA while it remained constant or fell slightly in C57Bl mice. It is evident that the antibody produced was not effective in controlling the infection nor did the antibody level correlate with survival in the longest surviving strains. Our results in BALB/c mice do not agree with those of Poels, van Niekerk & Pennings (1977) who found only traces of circulating antibody in BALB/c mice infected with *P. berghei* and suggested that all available antibody was quickly incorporated in immune complexes and removed from the circulation.

The antibody titre as measured by immunofluorescence gives a general indication of the host

humoral response but does not measure the quality of antibody produced. It has been shown in this laboratory (Perrin *et al.*, 1980) that more *P. falciparum* proteins are identified by sera from immune individuals with long-term malaria experience than by sera from patients recovering from a first infection. It is therefore possible that the antibody produced in the early stages of infection has little protective activity and that the quality improves with duration of infection.

Immune complex formation was a general phenomenon, observed in mice of all strains after day 6. The appearance of circulating complexes followed the natural sequence of antigen liberation and antibody production and was associated with complement consumption, as evidenced by reduction of C3 levels, in all except CBA mice which showed only a small transient depression of C3 between days 15 and 18, followed by recovery. The higher and sustained levels of immune complexes found in CBA mice may be due to failure to solubilize the complexes as a result of complement deficiency (Miller & Nussenzweig, 1975). Preliminary experiments in this laboratory (Aguado, personal communication) have shown that our CBA mice have less than 10% of the CH50 level found in other strains. It is not clear whether complement levels are related to susceptibility to *P. berghei*, since C57Bl mice, which have normal C levels, were as resistant as CBA mice. Susceptibility was greatest in A/J mice, which are deficient in C5.

Circulating immune complexes detected by the C1q-binding test include both IgG- and IgM-containing complexes. Additional studies carried out to identify the class of immunoglobulin present in the complement-reacting complexes showed strain differences in the profiles of these two forms of immune complex. IgM-containing complexes appeared earliest and were present in greatest amounts in BALB/c mice, whereas in CBA, IgM complexes occurred in lesser amounts and the level fell in later stages of infection. The level of IgG complexes, on the other hand, tended to rise throughout the course of infection in CBA mice while there was a reduction in the late stages in BALB/c and C57Bl mice,

In conclusion, this experiment provides new information on the host humoral immune response, the development of circulating immune complexes and the character of these complexes during the course of *P. berghei* infection. The level of complexes was not apparently related to susceptibility to the parasite. The results raise a number of questions, particularly regarding CBA mice, in which the immune response differed significantly from that in other mice in this experiment. It will be of interest to determine the importance of complement in this system, particularly in relation to the solubilization of circulating immune complexes. The antibody titre was greatest in CBA mice and the specificities of the antibodies produced in early and late infection may indicate which are especially involved in protection. Although the variation in susceptibility to the infection shown by the various strains could not be related directly to any single serological factor, pathological examination revealed a relationship between cerebral lesions and acute deaths in this model (Mackey *et al.*, 1980).

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