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ISOLATION AND ANALYSIS OF NEPHRICTIC-PRODUCING IMMUNE COMPLEXES IN PLASMODIUM BERGHEI-INFECTED MICE

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A nephritic condition was developed by infecting Swiss Webster albino mice with the malarial parasite Plasmodium berghei NK 65. These animals were tested for urinary protein and the presence of circulating immune complexes using reagent strips and a polyethylene glycol (PEG) precipitation assay. The circulating immune complexes were isolated from the sera using both affinity chromatography and PEG precipitation and from the kidney by acid elution. The isolated complexes were dissociated into their individual components and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The components of the complexes were transferred to nitrocellulose sheets and probed for the presence of malarial antigens using a rabbit anti-P berghei antisera. The overall humoral response to the malarial parasite was evaluated using a radial immunodiffusion assay. The present study confirmed that the malarial-infected animals not only developed the nephritic condition (as evident by the high levels of proteinuria) but also, as indicated by the PEG assay, have the presence of high levels of circulating immune complexes in their serum. The apparent absence in the SDS gels of any abnormal protein bands followed by the inability of the Western blot to reveal any malarial antigens provides some of the strongest evidence to date that these malarial proteins are not directly involved in the circulating immune complexes believed to be responsible for producing this nephritic condition. (J Natl Med Assoc. 1995;87:693-699.)

Key words • Plasmodium berghei • circulating immune complexes • nephritis

Over the past several years, a wealth of information has been published on the relationship between malarial infection and kidney disease. It is believed that the ensuing nephritic condition results from the deposition of circulating immune complexes into the glomerular basement membrane of the kidney.1 Many believe the presence of these immune complexes consists of not only immunoglobulins and complement components, but malarial antigens as well.2-5 However, since the suggestion that a mitogenic factor is responsible for the polyclonal stimulation of the lymphocytes,6 a number of reports have suggested that this intense production of antibodies has led to the production of anti-idiotypic antibodies that, when combined with its corresponding idiotype, leads to the formation of the circulating immune complexes believed to be responsible for the kidney damage.7-9

In this study, a different approach was taken than those previously reported to gain a better understanding as to the exact nature and composition of these malarial-induced circulating immune complexes. We used both affinity chromatography and polyethylene glycol (PEG) precipitation coupled with sodium do-
decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. In addition, a radial-immunodiffusion assay was used to analyze the overall humoral response to the malarial parasite.

MATERIALS AND METHODS
Experimental Animals
Swiss Webster albino mice, weighing an average of 21 g to 24 g, were quarantined for 7 days, housed in groups of three in wire-bottom cages, and fed commercial food prepared by the Purina Ralston Company (St Louis, Missouri). To ensure normal mouse body temperature, the mice were kept in a room with a constant temperature setting of 75°F. They were provided water ad libitum through an automatic watering system. The food containers were cleaned frequently to minimize algal growth and bacterial infection.

Parasitic Cells
Plasmodium berghei NK 65 (American Type Culture Collection, Rockville, Maryland) was used as the experimental organism. Cells were maintained in the laboratory by syringe passage in mice.

The malarial organisms were diluted with physiological saline in a red cell diluting pipet. The number of organisms per unit volume of saline was estimated using a hemacytometer. On day 1, the experimental animals were inoculated with 1×10⁴ infected cells.

Determination of Proteinuria
To determine if the mice had developed proteinuria, urine droplets expelled during cervical dislocation were collected using a capillary pipet and tested for the presence of protein using Albustix reagent strips (Fisher Scientific Co, Pittsburgh, Pennsylvania). The strips were dipped in the urine droplets and analyzed colorimetrically. The range was from 0 mg/dL (yellow) to 1000 mg/dL (blue-green). Amounts above 30 mg/dL were considered abnormal.

Polyethylene Glycol Precipitation
The presence of circulating immune complexes was determined using a PEG precipitation assay. Serum was obtained from animals by removing blood on the day of sacrifice, allowing it to clot for 2 hours at room temperature (27°C) and centrifuging it at 100 × g for 20 minutes. The sera from these animals then were incubated for 12 hours at 4°C with equal amounts of 8% PEG in borate-buffered saline (BBS) at pH 7.2. Following this incubation, the sera were analyzed visually for the presence of precipitation.

Isolation of Immune Complexes
Circulating immune complexes were purified from other serum proteins by applying sera from normal and infected mice to a 15×0.1 cm column, previously packed with Protein A-Sepharose CL-4B (Sigma Chemical, St Louis, Missouri). The affinity of the Protein-A for the Fc receptor on the IgG molecule allows for the binding of the immune complexes to the sepharose beads. Unabsorbed proteins were removed from the column by repeated washings with phosphate-buffered saline (PBS) (pH 7.2). The PBS was pumped through the column until the absorbance of the eluant, at 280 nm, was 0.02. At this time, those immune complexes that had absorbed to the affinity column were eluted with 1.0 M acetic acid (pH 3.0) and collected. The pH of these acid-eluted fractions was immediately raised to neutrality by the addition of 0.1 mL Tris base (1.0 M). Peak fractions, as determined by the absorbance at 280 nm, were pooled, dialyzed against PBS, and analyzed by SDS-PAGE.

Circulating immune complexes also were isolated from other serum components by centrifuging the precipitates from the PEG assay at 2000 × g for 20 minutes at 4°C. The supernatants were removed, and the precipitates were washed and resuspended in 3% PEG in PBS (pH 7.2). This material then was centrifuged at 200 × g for 20 minutes at 4°C. The supernatant was removed, and the precipitates were redissolved in equal amounts of PBS and incubated at 37°C for 1 hour. Following this incubation period, the complexes were dissociated and analyzed by SDS-PAGE.

Elution of Kidney Tissue
To analyze immunoglobulins and other proteins which had deposited in the kidney during infection, the kidneys were eluted according to the method described by George et al. Briefly, kidneys obtained from normal and infected mice were washed and homogenized in PBS (pH 7.2). This homogenate was centrifuged at 1500 g for 30 minutes at 4°C. The supernatant was discarded, and the proteins eluted from the kidney tissue by incubating the pellet, at 37°C for 2 hours, with 0.1 M citric acid (pH 2.5). This material was centrifuged at 3500 × g for 15 minutes, and the supernatant containing the eluted proteins, was titrated to pH 7.0 with sodium hydroxide. The eluted proteins were extensively dialyzed against PBS and analyzed by SDS-PAGE.

Polyacrylamide Gel Electrophoresis
Protein material isolated from both sera and renal
tissue was analyzed and compared using SDS-PAGE. For the SDS gels, a discontinuous system consisting of a 4% stacking gel and a 5% to 21% gradient separating gel was used. The electrophoresis run was performed in the Hoefer SE 600 Vertical Slab Gel Unit (Hoefer Scientific, San Francisco, California) at a constant current (30 mA) until the tracking dye reached the bottom of the gel. At the end of each run, the gels were either fixed and silver stained or transferred onto nitrocellulose membranes.

**Antibody Production**

*Plasmodium berghei* antigen, to be used in the production of an antisera in rabbits, was prepared according to the methods of Spira and Zuckermann. Briefly, blood collected from *P berghei*-infected mice by cardiac puncture was immediately mixed with 3.8% sodium citrate and washed by repeated centrifugation in PBS (pH 7.2). The erythrocytes were collected and lysed by incubation in 0.0001 M saponin (in PBS) for 15 minutes (37°C). The lysed material was centrifuged (3500 × g) for 3 minutes, and the parasite sediment was resuspended in fresh saponin for an additional 10 minutes. The free parasites then were washed (three to four times) by centrifugation (in PBS at 9000 × g). Finally, the parasites were centrifuged at 11 000 × g for 5 minutes, sedimenting them into a thick brown paste. This brown paste, consisting of pure parasites, then was homogenized and frozen at −40°C for 2 hours. On thawing, the homogenized material was centrifuged at 11 000 g for 3 minutes (4°C). The supernatant, which consisted of pure malarial antigen, then was used to produce an antisera in rabbits.

On day 1, each rabbit was given an intramuscular injection of 1.0 mL of malarial antigen in complete Freund’s adjuvant. Two weeks later, a booster injection consisting of 0.5 mL of antigen in incomplete Freund’s adjuvant was given intradermally. Two weeks after the booster, the animal was bled for serum from the malarial ear vein. This antisera was used to probe for malarial antigens.

**Western Blot Analysis**

To determine if any of the isolated proteins were produced by the malarial parasite, Western blot analysis was performed. The protein bands, isolated by SDS-PAGE, were transferred from the polyacrylamide gels onto Nitro-Plus nitrocellulose membranes (Fisher Scientific, Pittsburgh, Pennsylvania). These transferred proteins then were probed for malarial antigens using the anti-*P berghei* antisera, previously grown in rabbits. The transfer was done in the Transphor TE-52 Transfer Electrophoresis Unit (Hoefer Scientific, San Francisco, California). Each transfer took place in Towbin buffer using the following procedure: After the electrophoresis run, gels to be transferred were equilibrated for 30 minutes with Towbin buffer. Following equilibration, the gels were placed within the blotting cassette and transferred at 100 V for 2 hours. Following the transfer, the nitrocellulose sheets were removed from the transfer unit and probed for malarial antigens.

Nonspecific binding sites, on the nitrocellulose, were blocked by incubation for 2 hours in a blot buffer consisting of 3% Carnation milk (in PBS). Following blocking, the nitrocellulose was rinsed for 1 hour in a PBS-Tween buffer consisting of 0.05% Tween-20 in PBS (pH 7.2). The nitrocellulose then was incubated for 1 hour with a 1:1000 dilution of the rabbit anti-*P berghei* antibody. Following this incubation period, the nitrocellulose was rinsed in the PBS-Tween buffer and incubated for 1 hour with a secondary antibody consisting of biotinylated goat anti-rabbit IgG. After rinsing in PBS-Tween buffer, the nitrocellulose was incubated for approximately 30 minutes with a Streptavidin-horseradish peroxidase conjugate. Following a 30-minute rinsing in PBS-Tween buffer, the color was developed using 4-chloro-1-naphthol in 1% hydrogen peroxide.

**Radial Immunodiffusion**

To determine the concentrations of both IgM and IgG classes of immunoglobulins secreted during infection with *P berghei*, IgG and IgM single radial immunodiffusion (SRID) assays were performed. Single radial immunodiffusion plates (Melloy Laboratories, Springfield, Virginia), consisting of goat antimouse antibodies incorporated into agarose gel, were used. Serum from 0-, 7-, 14-, and 21-day infected animals were added to the wells and allowed to incubate for 24 hours. Following incubation, the diameter of the diffusion rings were measured with a millimeter ruler, and the concentrations determined by comparison against a set of known reference standards.

**RESULTS**

**Determination of Proteinuria**

The urinary protein concentrations of mice 7 days after infection with *P berghei* was 30 mg/dL (1 + proteinuria) (Figure 1). Animals sacrificed on days 14 and 21 showed a protein concentration of 300 mg/dL (3 + proteinuria). Control animals showed only trace amounts of protein in their urine (0 + proteinuria).
Polyethylene Glycol Precipitation

Visual analysis of immune complexes, precipitated by PEG, revealed only trace amounts of precipitation in the serum of mice 7 days after infection with P berghei. By days 14 and 21, there were increasing amounts of precipitation. Serum from control animals showed no visible signs of precipitation.

Polyacrylamide Gel Electrophoresis

SDS-PAGE analysis of immune complexes, isolated by Protein-A affinity chromatography, revealed protein bands ranging from 24 kd to 80 kd (Figure 2). In addition, these results showed that there was a massive increase in the amount of immunoglobulin material in the serum of the infected animals compared with the noninfected animals. In the case of the kidney, the banding pattern of the infected mice was similar to that of the noninfected mice. However, the infected kidney showed a large increase in the concentrations of those proteins eluted in the 24-kd to 45-kd range and in some instances showed an increase in proteins in the 55-kd and 25-kd ranges.

Analysis of immune complexes, isolated by the PEG precipitation method (Figure 3, lanes d and e), revealed a banding pattern similar to that observed in the Protein-A method. When comparing the banding pattern of normal and infected whole sera (lanes b and c, respectively), their patterns were also similar. However, the infected whole sera revealed a higher concentration of protein material in the 55-kd and the 25-kd ranges, whereas the normal whole sera showed a higher concentration of proteins in the 24-kd to 45-kd range.
Western Blot Analysis
Western blot analysis revealed that the rabbit anti-\textit{P. berghei} antisera failed to react to the proteins isolated from the normal and infected animals by either the Protein-A or PEG methods. When the same blots were performed using rabbit antimouse IgG as the antiserum, the results revealed strong reactivity in the 55-kd and 25-kd ranges. These molecular weight ranges are indicative of IgG heavy and light chains, respectively.

Radial Immunodiffusion Assay
The results of the radial immunodiffusion assay (Figure 4) revealed a steady increase in the concentration of IgG antibodies from days 7 to 21. In the case of IgG\textsubscript{1}, the increase was from 200 mg/dL to 520 mg/dL. The concentration of IgG\textsubscript{2a} increased from 50 mg/dL initially to 150 mg/dL by day 21. The IgG\textsubscript{2b} concentration increased from 90 mg/dL to 190 mg/dL. The immunoglobulin subclass IgG\textsubscript{3} increased from 400 mg/dL initially to 850 mg/dL by day 21. Immunoglobulins of the IgM class showed no significant increase, remaining steady at 10 mg/dL.

DISCUSSION
Previous reports have suggested that animals infected with malaria develop a condition known as glomerulonephritis.\textsuperscript{2,3} These reports also have suggested that this condition is due to the deposition of circulating antigen-antibody immune complexes into the glomerular basement membrane of the kidney. The present study confirms that these malarial-infected animals not only develop this nephritic condition (as evidenced by the high levels of proteinuria) but also, as indicated by the PEG assay, have the presence of high levels of circulating immune complexes in their sera. However, unless the malarial antigens were comigrating with the normal serum proteins, there was no visible evidence in the SDS gels of their involvement in these immune complexes. When probed by Western blot analysis for the possibility of comigrating malarial
antigens, the results also proved negative, providing even stronger evidence of the apparent absence of malarial antigens in the circulating immune complexes believed to be responsible for this nephritic condition.

The results of this study did show, however, patterns similar to those found in autoimmune diseases such as rheumatoid arthritis. Male et al.\textsuperscript{16,17} did an extensive analysis of the immune complexes deposited in the synovial fluids of patients with rheumatoid arthritis and revealed that IgG (peptide molecular weights of 52 kd and 25 kd), IgM (78 kd and 25 kd), and serum complement proteins (22 kd to 40 kd and 72 kd to 112 kd) were the predominant components of the immune complexes, and that IgG itself probably served as the primary antigen responsible for the pathological conditions.

In the present study, the predominant components of these complexes also appear to be IgG (MW 55 kd and 25 kd) and, in some cases, complement protein C1q (MW 22 kd to 30 kd) (Figure 2, lane c). In addition, Figure 2 (lane d) shows a high concentration of proteins (25-kd to 46-kd range) deposited in the infected kidney. The molecular weights of these proteins appear to be consistent with the serum complement proteins (C1q 22 kd to 30 kd; C3 40 kd to 46 kd; and C4 33 kd) found by Male et al.\textsuperscript{16,17} The concentrations of these same complement components appear to be lowered in the infected whole serum when compared with that of the noninfected whole serum (Figure 3, lanes b and c), and thus supports the belief that the deposited immune complexes caused activation of the complement system, leading to the deposition of these complement proteins into the glomerular basement membrane of the kidney. Consequently, as shown in this study, the infected animals will reveal a higher concentration of these complement components deposited in their kidneys, meanwhile leaving their serum levels of these same proteins severely depressed.

In addition, this study confirmed that these malarial-infected animals developed a hypergamma globulinemic state. This is evident in Figure 3, lane c, which shows a massive increase in IgG heavy and light chain proteins. However, the apparent absence of any malarial antigens associated with these antibodies suggests that some other factor or mechanism was responsible for the ensuing nephritic condition.

Greenwood\textsuperscript{9} first proposed that such a nonantigen-specific factor or mitogen was responsible for stimulation of the lymphocytes, leading to the intense production of antibodies. The present study not only adds support to this idea but is significant in that it provides definitive evidence of the apparent absence of malarial antigens in these nephritic-producing immune complexes.

Further evidence of this idea is provided by the use of the radial immunodiffusion assay, a technique commonly used to analyze the overall humoral response. It is known that the humoral system normally responds to a foreign antigen by first triggering an increase in the production of antibodies of the IgM class (primary immune response). This primary response is then followed by a secondary immune response in which there is an increase in the synthesis of antibodies of the IgG class. In this study, though, there was a massive increase in the synthesis of antibodies of the IgG class; unlike previous studies,\textsuperscript{1} there appeared to be no significant increase in the production of IgM antibodies (Figure 4). This suggests that the primary humoral response may not have been triggered. Consequently, the antibodies that were produced during this hypergamma globulinemic state appear not to be directed toward the malarial parasite. Therefore, it can be assumed that some other mechanism is responsible for this immune complex formation.

Jerne’s idiotypic network theory\textsuperscript{18} frequently has been used to explain this phenomenon. According to this theory, the immune system attempts to regulate itself by producing antibodies to its own immunoglobulins. Therefore, when this nonantigen-specific factor or mitogen stimulates the intense production of antibodies, as shown in this study, the immune system attempts to slow or regulate this activity by producing antibodies to its own immunoglobulins (anti-idiotypic antibodies). This leads to the production of circulating IgG-anti-IgG immune complexes that ultimately deposit in the glomerular basement of the kidney. Such anti-idiotypic antibodies have been proposed to be responsible for the production of circulating immune complexes found in animals infected with other parasites such as trypanosomes.\textsuperscript{7} The results of this study provide even stronger evidence of the possibility that the nephritic condition associated with malarial infections is a result of the glomerular deposition of these anti-idiotypic complexes as well.

\textbf{Literature Cited}


