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Detection of Circulating Immune Complex in Dogs Infected with *Babesia gibsoni*

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Immune complex glomerulonephritis in dogs and cats are recognized to occur with increasing frequency [18, 19]. In dogs, immune complex glomerulonephritis is associated with systemic lupus erythematosus [12], neoplasma [4], pyometra [15], multisystem involvement [9], infection with *Dirofiralia immitis* [1, 3] and adenovirus [13, 22].

Immune complex glomerulonephritis associated with hemospordian parasite infection has been well documented in the plasmodium species and babesia species. Annable et al. [2] reported that immune complex induced glomerulitis associated with *Babesia rodhaini* infection in rats. They demonstrated glomerular deposits of IgG and C3 and recovered proteins from involved kidneys by an acid elution which contained IgG against babesial antigen.

In dogs infected with *Babesia gibsoni*, however, it was reported that renal failure is not directly related to involvement of the babesial infection but is rather a secondary result that reflects a general failure of the kidneys caused by anemia [4, 6].

In this report we attempted to detect circulating immune complex (CIC) from dogs infected with *B. gibsoni* and as to whether the CIC cause immunological lesions to the kidneys.

Materials and Methods

1. *Babesia strain*

The strain of *B. gibsoni* used in this study was obtained originally from a naturally infected dog in Nagasaki prefecture in Japan. This strain was maintained in our laboratory by serial blood passage using mongrel dogs.

2. *Experimental dogs*

Nine healthy mongrel doges of 4~6 months old were vaccinated against canine distemper, canine parvovirus and infectious canine hepatitis and were

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also treated for intestinal parasites one month prior to the study. Then they were divided at random into two groups, 5 for infected and 4 for control groups, and kept in separate cages.

3. *Experimental infections*

Four dogs of control group were neither injected normal erythrocytes nor infected erythrocytes. Five dogs of infected group were inoculated intravenously with 1×10^8 parasitized erythrocytes per 1 kg body weight. The erythrocytes were collected from a splenectomized dog in which 15% of the erythrocytes contained *B. gibsoni*. Observations were carried out to the day immediately following the peak of parasitemia for 1 dog and to the 30th day after infection for the remaining 4 dogs. The dogs were then sacrificed by sodium pentobarbital injection and necropsied.

4. *Hematological examinations*

Blood samples were collected at three-day intervals in both groups. The following tests were performed: Packed cell volume (capillary tube method), erythrocytes count (counting chamber method), numbers of parasites (Giemsa stain of blood smear), BUN (urease-indophenol method), serum creatinine (Folin-Wu method) and γ-globulin (electrophoresis).

The CIC in the serum was detected by using the 7% polyethylene glycol (PEG-6000) precipitation method [23].

5. *Immunopathological examination*

(1) Light microscopical examinations. For light microscopy, 3 μm thick sections were cut from paraffin blocks prepared for direct immunofluorescent test and stained with either haematoxylin eosin (HE) and periodic acid-methenamine silver stain (PAM).

(2) Direct immunofluorescence. At the time of necropsy, 2 mm thick samples of kidneys were fixed immediately in cold 95% ethylalcohol and embedded in paraffin by the method reported previously [10, 21]. Fine 3 μm thick sections were incubated with FITC-conjugated anti-dog IgG (Miles-Yeda, Ltd.) and the degree of IgG deposits in the glomerular basement membranes (GBM) and the tubular basement membranes (TBM) were shown as the intensity of IFA staining. The intensity of C3 deposits was also observed as the method for IgG using FITC-conjugated anti-dog C3 (Cooper Diagnostic Inc.).

(3) Indirect immunofluorescence. The indirect fluorescent antibody technique was applied to detect specific antibody titers in serum by the method reported by Tamura et al. [20] and in the kidney eluates.

6. *Antibody-elution from the kidneys*

Antibody-elution was performed on the kidneys of 3 infected and 2

control dogs. The kidneys were frozen at −80°C until antibody-elution by method reported previously [11] with the following modifications. The homogenized kidneys in 0.15 M PBS (pH 7.2) were washed as many times as necessary until the supernatant fluid has no discernible proteins by UV absorption at 280 nm. Elution with 0.02 M citrate buffer (pH 3.2) was carried out for 16 hours at 4°C with constant stirring. The eluate was subsequently neutralized with 0.1 N NaOH, filtered through a 0.25 mm Millipore prefilter, dialyzed against PBS for 2 days and concentrated by PEG-6000 to a protein concentration of 10 mg/m . The eluate was used in an indirect fluorescent antibody test against blood smears containing *B. gibsoni*.

Results

1. Hematological and immunopathological findings of experimental dogs

All infected dogs exhibited the onset of the disease but none of them died until the 30th day post infection (p. i.), except one dog that was euthanized on the 12th day p. i. (Fig. 1).

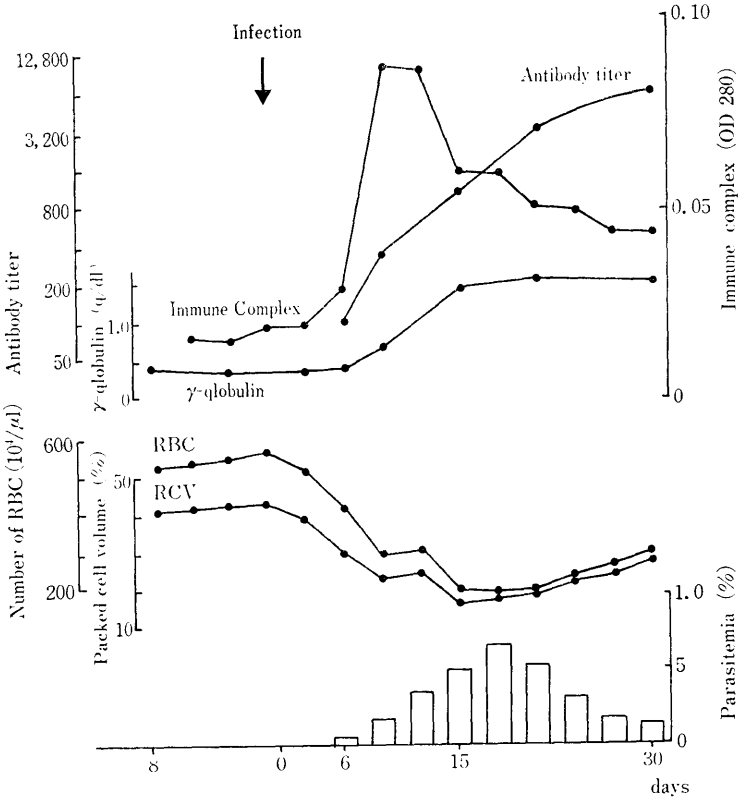

Fig. 1. The mean value of the observation items in the group infected with *B. gibsoni*.

Parasites first appeared in the peripheral blood on the 3rd to the 6th day p. i., and parsitemia reached a maximum level of 6.6% on the 18th day p. i. and then gradually decreased. Packed cell volume and erythrocyte count decreased with parasitemia, showing a minimum level at 0 to 3 days before the maximum parasitemia, and then gradually increased. Gamma globulins increased with parasitemia, then maintained a level as high as 1.5 g/100 ml until the end of the experiment. Antibody titers began to increase from the 6th day p. i., showing an average of 1 · 6400 on the 30th day p. i.. CIC began to rise rapidly from the 6th day p. i., showing a maximum level of 0.087 (OD 280) between the 9th day and the 12th day p. i.. And even on the 30th day p. i., CIC showed a high level of 0.044 (OD 280) compared to 0.020 (OD 280) on the preinfection day, which represented a significant increase ($P<0.01$).

In the control group, however, changes in erythrocytes count, packed cell volume, γ-globulins and CIC were within the normal range.

Throughout the observation period, renal failures in the infected group and control group were not observed as far as findings in BUN and serum creatinine levels were concerned (Fig. 2).

Thickening of the GBM in dogs in both groups was not observed by HE and PAM stain. But the immunofluorescence test demonstrated a significant increase in the deposits of IgG along the GBM, which were in a

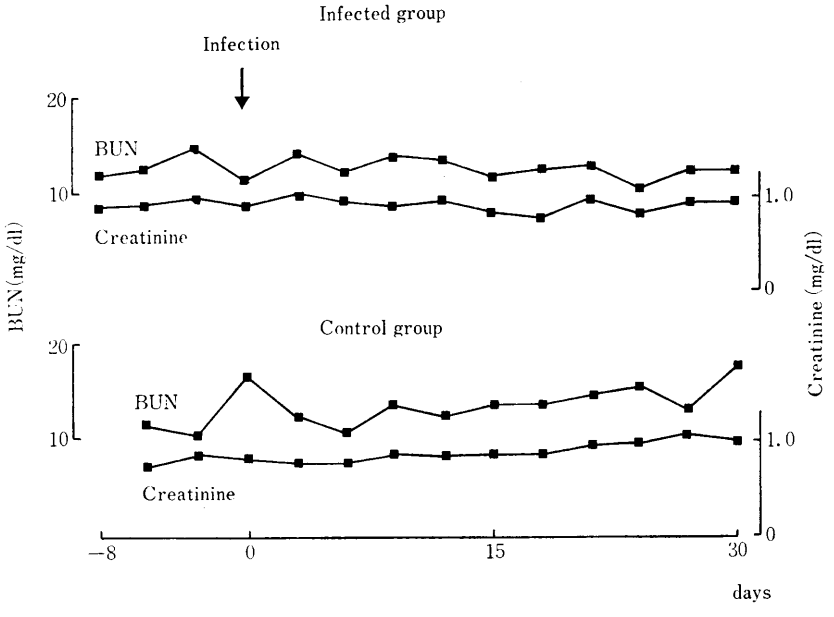

Fig. 2. Mean value of BUN and serum creatinine level in the group infected with *B. gibsoni* and the control group.

granular pattern, in the infected group (Figs 3, 4) as compared with the deposits of the control group (Figs 3, 5). The deposits of IgG along the TBM were observed as a linear pattern in both groups and there was no significant difference between them. C3 was found along the GBM in a

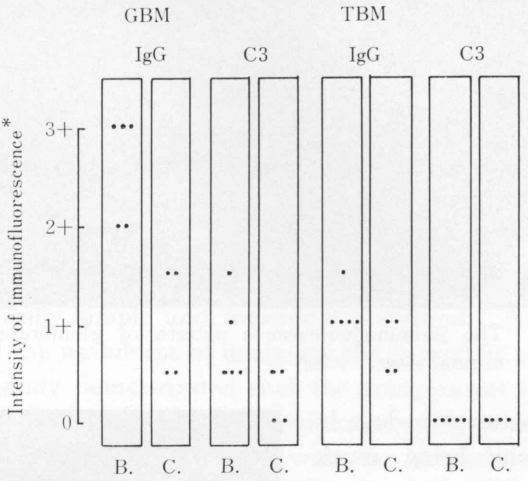

Fig. 3. Intensity of IFM staining for IgG and C3.

GBM: Glemerular basement membranes
TBM: Tubular basement menbranes
B.: Babesiosis C.: Control •: indicates a dog
* The intensity of immunofluorescent staining was semiquantitatively greaded: 0, no staining; 0.5+, slightly higher fluorescent intensity than background; 1+, trace or small amount of staining; 1.5+, higher fluorescent intensity than 1+; 2+, moderate staining; 3+, bright staining.

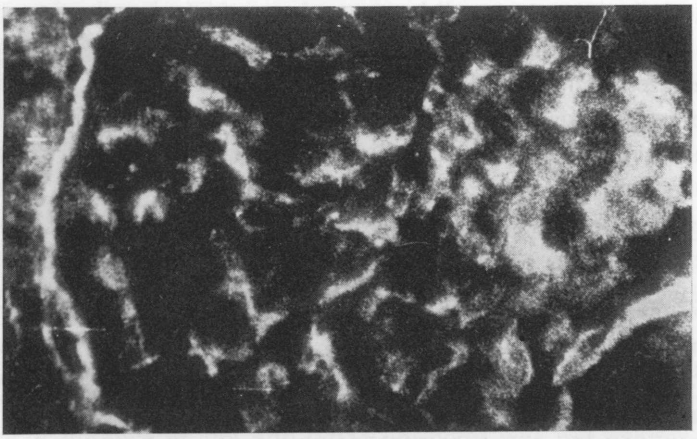

Fig. 4. The immunofluorescent pattern of glomerulus from the dogs infected with *B. gibsoni*. Deposition of IgG along the glomerular basement membrane is indicated. ×800

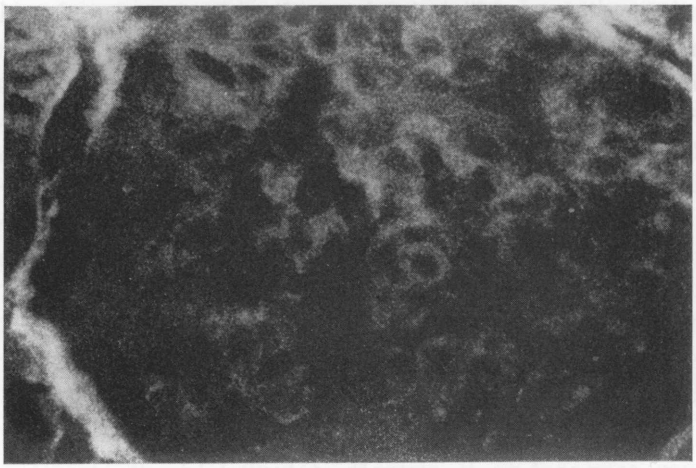

Fig. 5. The immunofluorescence pattern of glomerulus from normal dogs. ×800

diffuse granular pattern in dogs in the infected group, being much less intense and diffusely localized than that for IgG. Deposits of C3 along the TBM, however, were not observed in dogs in both groups. The results of the comparison made of the deposits of IgG and C3 in the kidneys at 12 days p. i. and 30 days p. i. revealed no difference in their intensity and localization.

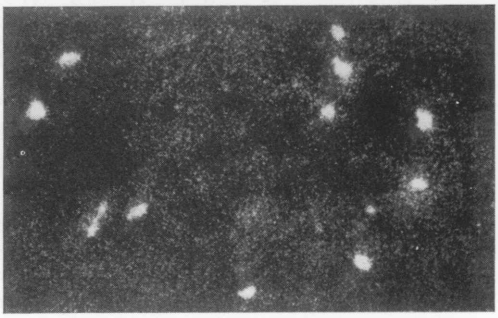

Fig. 6. The immunofluorescent pattern of parasitized erythrocytes stained with antibody eluted from the kidneys of dogs infected with *B. gibsoni*.

The eluate obtained from kidneys of infected dogs showed reactivity to *B. gibsoni* antigens by indirect immunofluorescence (Fig. 6). However, the eluate obtained from the control kidneys failed to react with *B. gibsoni*.

Discussion

In the previous observations of dogs infected with *B. gibsoni*, Fowler et al. [4] reported that all infected dogs died within 20 days following infection, and the BUN level was in the normal range until the terminal stage and increased rapidly during the last 4 to 6 days prior to death. Halliwell et al. [5] reported that obvious BUN increase occurred not at the early stage but at the terminal stage of glomerulonephritis. Hoe et al. [8] also noted that increase in BUN is not found in all cases of nephritic dogs. In the

present study, increase in BUN and serum creatinine did not occur within the observation period. Slauson et al. [19] suggested that the granular deposition of IgG and C3 indicated immune complex glomerulonephritis. In a study of rats infected with *B. rodhaini*, Annable et al. [2] observed the presence of moderate, acute prologerative glomerulities with deposits of IgG and C3 arranged in a granular pattern. In this experiment of *B. gibsoni* infection, the granular deposits of IgG and C3 found along the GBM agreed with the findings of previous reports [7, 16, 17, 21, 24] regarding the pattern of CIC deposition. These glomerular changes were considered to reflect the early stages of membranous glomerulonephritis, although glomerulonephritis was not demonstrated by the preparations of HE and PAM stain in this experiment.

For the detection of CIC, the PEG precipitation assay has been promoted as a technically simple and inexpensive method. Annable et al. [2], however, reported the usefulness of analysing the hemolytic complement levels (CH 50). Their study demonstrated that the complement levels fell sharply during parasitemia and remained depressed for a week, which indicated that the complement consumption occurred with the formation of CIC in the serum. In other words, changes in CIC level are inversely proportional to those at the complement level. This relation was substantiated by the findings of CIC level detected by PEG in the present study. The level of CIC showed a significant high level even at the end of the experiment, as compared to the level prior to the infection.

Annable et al. [2] also suggested that maximum deposition of immune complex from the circulation occurred during period of continuing high level of CIC. In the present experiment, the degree deposition of IgG and C3 in the glomerulus in the kidneys from one dog at 2 days after peak parasitemia was compared with that in the kidneys of a dog from 4 days to 12 days following peak parasitemia with the result that no outstanding differences were observed between them. This failure to demonstrate differences in the degree of CIC deposition seemed to be caused by the fact that the maximum CIC levels in the serum preceded the peak of parasitemia and immune complex deposition already occurred at the period of maximum parasitemia. Regarding antibody production, it was also assumed that there was no difference of CIC deposition in the glomerulus when antibody titers were still rising.

The immunopathological nature of the renal deposits was substantiated by the presence of antibodies which react with *B. gibsoni* antigen. Although the presence or absence of the anti-GBM antibody was not examined in this experiment, it was considered that the antibody was not present by the deposition pattern of IgG andC3 [14].

It was therefore concluded that the formation of CIC in the serum and subsequent deposition in the GBM occurred during the course of *B. gibsoni* infection.

Summary

Circulating immune complex (CIC) from dogs infected with *Babesia gibsoni* were detected by using polyethyelen glycol precipitation method. CIC increased rapidly from the 6th day, and reached maximum levels between the 9th day and the 12th day after inocultaion. CIC showed high levels even on the 30th day after inoculation compared to that on preinfection. BUN and serum creatinine levels were within the normal range in all infected dogs. Histological examinations of the kidneys did not show obvious lesions on the glomerulus. Immunofluorescent observation revealed deposits of IgG and relatively slight deposition of third complement (C3) along the gromerular basement membranes (GBM). The eluate obtained from the kidneys of the infected dogs showed reactivity to the smears containing *B. gibsoni*.

Although glomerulonephritis was not observed, it was therefore clarified that the formation of CIC in the serum and subsequent deposition in the GBM occurred during the course of *B. gibsoni* infection.

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要　　約

Babesia gibsoni 感染犬の血清からポリエチレングリコールを用いる方法で免疫複合体の検出を試みた。接種18日後に *parasitemia* はピークに達し，蛍光抗体間接法による抗体価は30日後に6,400倍に達した。血清中の免疫複合体 (CIC) は，原虫の血中出現に伴い上昇し，30日後においても接種前より高い値を示した。血中の BUN とクレアチニン濃度は，正常値範囲内で推移し，腎糸球体に組織学的な変化は認められなかった。しかし *B. gibsoni* 感染犬の腎糸球体において蛍光抗体間接法により IgG と C_3 の糸球体基底膜に沿った沈着が認められ，また感染犬の腎からの酸抽出蛋白は，*B. gibsoni* 抗原と反応した。以上の結果から糸球体腎炎にはいたらなかったが *B. gibsoni* 感染症の経過中に，原虫抗原に由来する CIC が血中に形成され，腎糸球体に沈着することが明らかになった。