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Circulating Antigens and Immune Complexes in the Serum of Rat Infected with *Angiostrongylus cantonensis*

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In some protozoan and helminthic infections in the host blood stream, soluble antigen of the parasite origin has frequently detected in the circulation of infected hosts (Gold *et al.*, 1966; D'Alesandro, 1972; Smith *et al.*, 1972; Hirata and Akusawa, 1975; Deelder *et al.*, 1976; Hirata, 1976) and the possible use as a reliable immunodiagnostic tool has been pointed out (Smith *et al.*, 1972; Phillips and Draper, 1975). It was suggested that the circulating antigens were detected as the result of insufficient antibody formation of host because of their weak antigenicity, any defect in immune response or others. By unbalance of antigen/antibody ratio, the antigens extricated into the circulation could not be rapidly eliminated and they persisted as circulating antigens for a long time. For example, malaria S-antigens, often found in malarious plasma, were considered to be modified erythrocytic components (Wilson, 1974). Circulating antigen originated from schistosome gut was polysaccharide and its antigenicity appeared to be considerably weak (Nash *et. al*,, 1974; Nash, 1974; Lichitenberg *et al.*, 1974). On the other hand, it was considered that antigens of parasite origin, in general, induced specific antibody production of host and then being eliminated from the circulation. In chronic blood parasite infections, some of antigens originated from parasites are considered to react to specific antibodies and form immune complexes. In fact, in malarial, trypanosomal and schistosomal infections, circulating immune complexes were demonstrated in the patients and experimental animals using various methods (Lambert and Houba, 1974; Phillips and Draper, 1975; Houba *et al.*, 1976; Krettli *et al.*, 1976; Bout *et al.*, 1977; Jones *et al.*, 1977). It was pointed out that the formation of such immune complexes may reduce direct attack of antibody to the parasite and block the induction of other immunities mediated by specific antibody (Cohen, 1976; Butterworth *et al.*, 1977). In addition to these roles, recently, the inflammatory tissue lesions mediated by immune complexes have attracted attention in some parasitic diseases (Andrade *et al.*, 1971; Houba, 1977; Queiroz *et al.*, 1973; Lambert and Houba, 1974; WHO-Report, 1977; Blackett and Ngu, 1976; Poltera *et al.*, 1976).

In the present study, the authors examined if the circulating antigen or immune complexes could be detected in the sera of rats infected with *Angiostrongylus cantonensis*.

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Materials and Methods

1) *A. cantonensis*

The strain of *A. cantonensis* used was first obtained from giant African snails, *Achatina*

fulica, on Yoron-Jima, one of the Amami Islands (Yamashita *et al.*, 1978) and it has been maintained in our laboratory by the passage through aquatic snails, *Biomphalaria glabrata*, and albino rats, *Rattus norvegicus*, since 1977.

2) The 3rd-stage larvae and infection in rats

The snails, *B. glabrata*, 60 days after infection with the 1st-stage larvae from feces of infected rat, were incised into pices and digested with the artifical digestive juice (0.1 % pepsin, 0.7% HCl) at 37 C for one hour. The digested material was filtered through a stainless-steel sieve, the filtrates were allowed to sediment and vigorously motile 3rd-stage larvae in the sediments were counted under a binocular microscope. Fifty or 100 larvae were orally administered to a rat with a stomach tube. The blood was taken from a tail vein of the infected rat and the serum was separated immediately after coagulation at 37 C for 3 hours without being preserved at lower temperature to prevent the euglobulin precipitation.

3) Whole worm extract

On 50th day of the infection, adult worms were recovered from the pulmonary artery of the rats and were washed 3 times with sterilized saline, ground and then lyophilized. An excess of cold acetone was added to the dried mass for delipidization, and the mixture was stirred for 10 minutes and centrifuged at 8,000 G for 30 minutes. Then, phosphate buffered saline (PBS), pH 7.2 was added to the sediment and well stirred for 48 hours at 4 C using a magnetic stirrer. After this, the mixture was centrifuged at 8,000 G for 30 minutes and the supernatant fluid after adjustment of its protein concentration to 20 mg/ml was used as a whole worm extract antigen (WWE).

4) Preparation of antiserum

To obtain antiserum against WWE, the rats weighing ca. 300 g were used. As an immunization method, intramuscular and subcutaneous injections of 2 ml of equivalent mixture with WWE and Freund's complete adjuvant were made into a rat. The similar immunizations were made 5 times at interval of 10 days and the antiserum (Anti-WWE) was obtained 2 weeks after the last immunization.

The antiserum against serum components precipitating with 6.0% polyethylene glycol (PEG) was obtained as described below. The rat serum, 50 days after the infection, was added to an equal volume of 12.0% PEG dissolved in borate buffer (pH 9.3, μ=0.1), and the precipitates (PEG-ppt) formed under the final concentration of 6.0% PEG were collected by centrifugation at 1,500 G for 20 minutes and dissolved in PBS of the initial serum volume. The dissolved PEG-ppt was injected together with Freund's complete adjuvant into a rabbit weighing 2.0 kg by the similar immunization schedule as mentioned above. The serum from the rabbit was used as the antiserum (Anti-PEG-ppt).

5) Detection of immune complexes

According to Creighton *et al.* (1973), nonspecific precipitation method by PEG was used for detection of immune complexes in the serum. In the present study, the final concentration of PEG was 6.0% based on the observation of result mentioned later. One volume of infected rat serum was mixed with 1 volume of 12.0% PEG, and was left at 4 C for 12 hours. Then, the mixture was centrifuged at 1,500 G for 20 minutes and the PEG-ppt was dissolved with PBS of initial serum volume. The PEG-ppt levels were determined by ultraviolet spectrophotometry at 280 nm (Digeon *et al.*, 1977; Bout, 1977) after 50-fold dilution with 0.1 N NaOH solution.

6) Analysis of antigen in immune complexes

Detection of *A. cantonensis* antigen from immune complexes was done by the following two methods. The one was that PEG-ppt obtained from sera of rats 50 days after infection was dissolved in PBS and injected into normal rats using the same immunization schedule as previously stated. Then, it was examined by double diffusion method whether antibody against *A. cantonensis* antigen was formed or not. The other,

according to Phillips and Draper (1975), PEG-ppt was dissolved in citrate buffer solution (0.02 M, pH 3.2) equivalent in volume to initial serum, and it was allowed to stand for 12 hours at 4 C for dissociation of antigen-antibody complexes. Then, double diffusion method was done to detect antigen between Anti-WWE and the dissolved solution. Furthermore, the antigenic substances were analized with Anti-PEG-ppt immunoelectrophoretically.

7) Double diffusion and immunoelectrophoresis

Double diffusion method was performed according to the method of Ouchterlony (1962) using 1.2% agarose (Behring Institut) in veronal-HCl buffer (pH 8.6, μ=0.05). On a 5.0×8.0 cm glass slide, thin agarose gel-plate with 1.5 mm thickness was prepared and wells with 6.0 or 12.0 mm diameter were formed on the plate. The distance between the wells was 5.0 mm. Each well was filled with antibody or antigen solution, and the plate was allowed to develop precipitin band at 4 C for 48 hours. After this, the plate was washed with the veronal-HCl buffer for 3 days, dried under filter paper and stained with amidoblack 10 B.

Immunoelectrophoresis used was the micro-method of Scheidegger (1955) using the same gel-plate as above. Antigen solution was added to well with 4.0 mm diameter and the potential of 35 V was maintained across the slide for 4 hours. After the electrophoresis, antibody solution was filled in the trough formed across the slide at 5.0 mm distance from the well and precipitin bands were allowed to develop for 48 hours. The washing, drying and staining procedures were similar to the above.

8) Cyclophosphamide treatment of the rat

Some of the infected rats were treated with cyclophosphamide (CPA) (Endoxan : Shionogi Co. Ltd.) to suppress host immune responses. Thirty six mg of cyclophosphamide per Kg of rat was intraperitoneally injected into the animal twice 2 and 10 days before and after infection.

Results

1) Detection of antigen in infected rat sera

Sera from 20 untreated and 6 CPA-treated rats infected with 100 larvae were directly examined in the presence of circulating antigen by double diffusion method with Anti-WWE. The sera were taken every 10 days after infection for 60 days and adult worms were recovered 60 days after infection. As shown in Table 1, the sera of 4 (20.0%) out of 20 untreated and 2 (33.3%) out of 6 CPA treated rats indicated the occurrence of antigen during the course of the infection. The antigen was demonstrated in the sera of 3 and 1 untreated rats 50 and 60 days after infection respectively. While, in the CPA-treated rats, one was antigen-positive in 10 days and the other one was positive

Table 1 Demonstration of antigen in the sera of 4 rats without previous treatment and 2 immunosuppressed rats infected with 100 larvae of *A. cantonensis*

Rat No.	Infected rat	Days after infection						Number of worms recovered from lungs		
		10	20	30	40	50	60	Male	Female	Total
1	No treatment	−	−	−	−	+	−	19	26	45
2	〃	−	−	−	−	±	−	17	33	50
3	〃	−	−	−	−	+	−	37	43	80
4	〃	−	−	−	−	−	+	29	24	53
5	*Immunosuppressed	+	−	−	−	−	−	24	26	50
6	〃	−	+	+	−	−	−	20	28	48

* Immunosuppressed group of rats were administered with cyclophosphamide prior to and after parasite infection. See materials and methods.

in the two sera obtained 20 and 30 days after infection. Like these, detection of antigen in the infected rat sera was temporary excluding the latest one. The precipitin bands were considerably weak in the all of 6 rats. Average number of the adult worms in the pulmonary artery was 50.8, ranging from 36 to 80, but no correlation between the number of parasitized worms and detection of antigen in the serum could be observed.

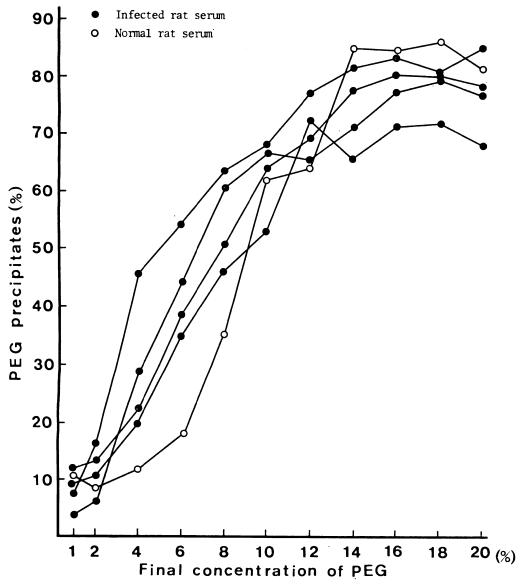

Fig. 1 Solubility of the infected and normal rat sera at various PEG concentrations.

2) Determination of PEG concentration for detection of immune complexes

In order to decide PEG concentration for detection of immune complexes, sera of 4 rats 50 days after infection and an uninfected normal rat were examined in solubility in various PEG concentrations from 1.0 to 20.0%. The result is shown in Fig. 1. The precipitates of infected rat sera were much more than those of normal serum in the final PEG concentration from 4.0 to 8.0% but large differences between the two were observed in 4.0 and 6.0% of PEG concentrations. In general, as PEG concentration becomes higher, proteines other than immune complexes are known to become precipitated. In the present examination, precipitates of normal rat serum tended remarkably to increase in more than 10.0%. From the result, PEG concentrations from 4.0 to 8.0% was thought to be adequate to detect and assay immune complexes in rat serum. Therefore, the final concentration of 6.0% was used in

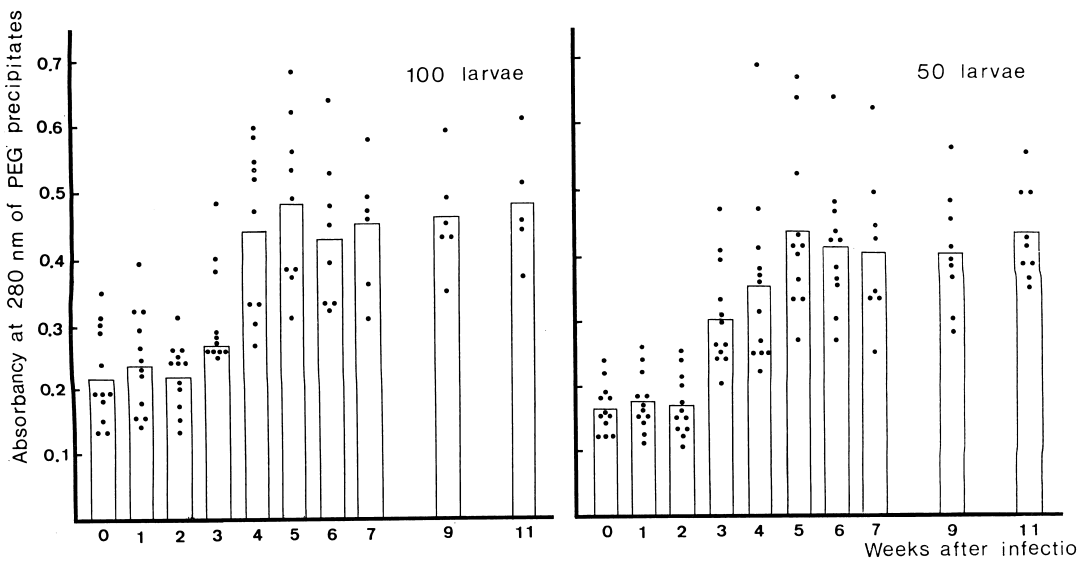

Fig. 2 Periodical transition of PEG precipitates levels in sera of rats infected with 100 or 50 larvae of *A. cantonensis*.

the later experiments.

3) Increase of PEG-ppt in infected rat sera

Transition of PEG-ppt in the sera collected weekly from two groups consisting of 12 rats infected with 50 and 100 larvae is shown by individual and by mean levels in Fig. 2. In both groups, PEG-ppt did not increase in levels within 2 weeks after infection but they distinctly increased during 3 to 4 weeks. Subsequently, the average of PEG-ppt levels did not so differ in the two groups, and maintained high levels in each group from 4 to 11 weeks as compared with those of rats before infection. The PEG-ppt levels in rats infected with 50 larvae were slightly lower in the mean value every week than those of 100-infected rats, excluding one 3 weeks after infection.

4) Detection of *A. cantonensis* antigen in PEG-ppt

To determine whether antigenic components of the parasite existed in the PEG-ppt, double diffusion method was done between the antiserum of rat immunized with the PEG-ppt as previously stated and WWE. As seen in Fig. 3, one precipitin band was demonstrated and it was identical with one of the bands developed between WWE and infected rat serum or Anti-WWE used as control.

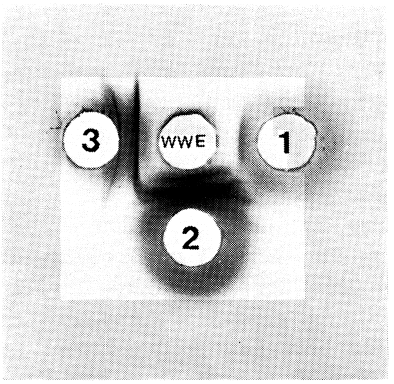

Fig. 3 Demonstration of antigenic components of *A. cantonensis* in PEG precipitates with the antiserum of rat immunized with PEG precipitates.
WWE: whole worm extract of *A. cantonensis*; Wells 1, 2 and 3 were filled with antiserum of rat immunized with PEG precipitates, infected rat serum 50 days after infection and Anti-WWE respectively.

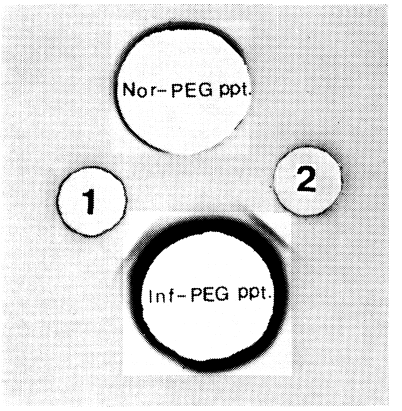

Fig. 4 Detection of antigenic components of *A. cantonensis* in PEG precipitates dissolved in citrate buffer pH 3.2. Wells 1 and 2 were filled with Anti-WWE and antiserum of rat immunized with PEG precipitates respectively.
Nor-PEG ppt: PEG precipitates from normal rat serum; Inf-PEG ppt: PEG precipitates from infected rat serum.

Further to directly detect the antigen, the PEG-ppt from sera of rats 40 days after infection were dissolved in citrate buffer, pH 3.2 and examined in development of precipitin band with Anti-WWE by the double diffusion method. As shown in Fig. 4, one definite precipitin band could be seen between the dissolved PEG-ppt and Anti-WWE. Similar precipitin band was also developed by the above antiserum against the PEG-ppt. No precipitin band, however, could be observed between the dissolved PEG-ppt from normal rat serum and Anti-WWE.

5) Immunoelectrophoretic analysis of PEG-ppt

The components in the PEG-ppt were analized immunoelectrophoretically with Anti-PEG-ppt obtained from an immunized rabbit and the result is shown in Fig. 5. Anti-PEG-ppt developed 4 precipitin bands with each of WWE and normal rat serum. Among the 4 bands with normal rat serum,

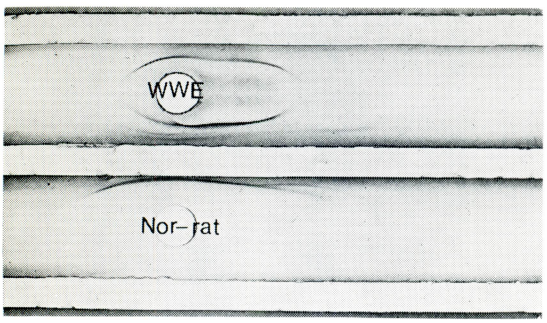

Fig. 5 Immunoelectrophoretic analysis of PEG precipitates by rabbit serum immunized with PEG precipitates of infected rat sera. Nor-rat : normal rat serum ; WWE : whole worm extract of *A. cantonensis* ; Median trough was filled with rabbit anti-PEG precipitates antiserum and other troughs with the anti-PEG precipitates absorbed with normal rat serum.

a major band recognized to be developed with rat IgG. When Anti-PEG-ppt serum was absorved with normal rat serum, all bands with rat serum did not observed but 2 out of 4 bands with WWE recognized after the absorption.

Discussion

In the present study, the authors carried out an experiment to detect the circulating antigen and immune complexes in sera of rats heavily infected with *A. cantonensis*. As result, the circulating antigen was faintly recognized in 4 (20.0%) out of 20 untreated and 2 (33.3%) out of 6 CPA-treated rats infected with 100 larvae, and no noticeable difference of detection rate of antigen was found in the two groups of rats. Following consideration will reasonably be made to explain this finding. CPA-treatment caused a remarkable increase of fatality rate of rats when enough amount of the drug was given to them to suppress antibody production in the rats, and the effect of CPA-treatment on antibody formation by double diffusion method was that it was shown 10 days later than in untreated rats. Contrary to this observation, soluble antigen was demonstrated in the group of rats subjected to CPA-treatment at earlier stage of infection (10, 20 and 30 days) than that shown in untreated rats (50 and 60 days after infection).

Circulating antigens have clearly been demonstrated in malaria, trypanosome and schistosome infections (Weitz, 1960 ; Gold *et al.*, 1969 ; D'Alesandro, 1972 ; Eaton, 1939 ; Deelder *et al.*, 1976 ; Hillyer, 1976 ; Hirata, 1976). In malaria and trypanosome infections, the parasites may proliferate in the host and the detection of circulating antigen appeared to be related to the parasite number and developing stage during the course of infection (Eaton, 1939 ; Weitz, 1960 ; Wilson and Bartholomew, 1975). In the case of schistosomiasis, the antigen detected in the circulation was a polysaccharide contained in a large amount of "schistosome vomitus" (Bawden and Weller, 1974 ; Nash *et al.*, 1974 ; Lichtenberg *et al.*, 1974). It is weakly antigenic substance and is difficult to remove from circulation during short period (Nash *et al.*, 1974 ; Hillyer, 1976). In the present study, biochemical research of antigen detected in the sera of infected rats was not examined. More highly sensitive detection method of antigen and preparation of highly responsible antiserum to the antigen are considered to be necessary for the further examinations.

On the other hand, PEG precipitates in the sera of infected rats increased after infection and high levels were maintained during from 4 to 11 weeks after infection, which suggested the presence of immune complexes in their circulation. The PEG precipitate levels in the group of rats infected with 100 larvae were slightly higher than in 50-infected group. To explain the small difference between the two groups, it may be possible to consider that the PEG precipitate levels reached to a plateau 5 weeks after infection. The commencement of the female egg-laying and release of antigens from the 1 st-stage larvae may be related to the small difference in the levels of PEG-ppt. Furthermore, PEG precipitation method used in this study was nonspecific, so that the precipitation of various immune com-

plexes was supposed to be simultaneous. However, from the increase of precipitate levels after infection, the immune complexes after rapid reaction of parasite antigens to specific antibodies were reasonably suggested to be formed. Similarly, in human and murine schistosomiasis, large difference was observed between PEG precipitate levels in the infected and in the uninfected animals which suggested the presence of circulating immune complexes in the infected hosts (Bout *et al.*, 1977; Digeon *et al.*, 1979).

In order to ascertain the above-mentioned suggestion, detection of parasite antigen to bind itself to antibody was attempted using the following two techniques: (a) Normal rats were immunized with PEG-ppt obtained from infected rats and examined whether antibody against *A. cantonensis* antigen could be produced in the rats. (b) Acidification of PEG-ppt from infected rats was followed by double diffusion method with Anti-WWE to measure the presence uncombined antigen. From these results, the substance seemed to be originated from the parasite could be demonstrated in the PEG-ppt. Two precipitin bands were also demonstrated by the immunoelectrophoretic examination between WWE and Anti-PEG-ppt from a rabbit immunized with the PEG-ppt when the antiserum was absorbed with normal rat serum.

It has been suggested that, in some cases, immunopathological mechanisms are involved in the pathogenesis of lesions produced by some parasitic diseases. As one of the mechanisms, in malaria, trypanosomiasis and schistosomiasis, it was presumed that soluble immune complexes are formed in circulation and subsequently deposited in the vessel wall of target organs with high filtering capacity. Most of such immune complexe type of lesions were concerned to glomerular lesions (Ward and Kebukamusoke, 1969; Houba *et al.*, 1971; Boonpucknavig *et al.*, 1973; Ehrlich and Voller, 1972; Lambert and Houba, 1974; Nagle *et al.*, 1974; Andrade *et al.*, 1971; Queiroz, *et al.*, 1973; Moriearty and Brito, 1977). In trypanosomiasis, the following possibility was described that immune complexes formed in the extravascular spaces might play a major role in the development of lesions in myocardium and brain tissue (Blackett and Ngu, 1976; Poltera *et al.*, 1976).

Based on the present results, pathogenicity of the immune complexes should be investigated in relation to worm number survived in host and the antibody formation in the near future.

Summary

Examination results of antigenic substances and immune complexes in the circulation of rats experimentally infected with *A. cantonensis* were as follows.

1) By double diffusion method, the antigenic substances were demonstrated in 20.0% of untreated and 33.3% of cyclophosphamide treatment rats infected with 100 larvae. The appearance of the substances, however, was transitional.

2) Six % PEG precipitate levels in the infected rat sera were remarkably increased 4 weeks after the infection and persisted at plateau level afterwards.

3) In the PEG precipitates, at least 2 kinds of the parasite antigens could be demonstrated by immunoelectrophoresis.

From those results, it was considered that the antigens were released from the parasite into the host blood stream and that they were thought to form immune complexes after binding then to specific antibodies.

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広東住血線虫感染ラット血清中における循環抗原および免疫複合体

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広東住血線虫感染ラット流血中における本線虫由来の抗原物質および免疫複合体の検出を試みた結果，以下の成績を得た．

1) 二重拡散法により，正常感染ラット群では20％の個体に，免疫抑制を行なつた感染ラットでは33.3％の個体にそれぞれ抗原が検出され，その時期は一過性であつた．

2) 感染ラット血清における6％ PEG の存在下での沈澱物量は感染後4週目より著明に増加し，その後，平衡状態であつた．

3) PEG 沈澱物中には本線虫由来の抗原物質が検出され，その中には少なくとも二種類の抗原物質の関与が示された．

以上の結果より，感染ラット流血中には本線虫由来の抗原物質が遊離されるが，これらは血中の特異抗体と反応して免疫複合体を形成していることが示唆された．