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Antigenic Analysis of the Peritoneal Exudate of *Toxoplasma gondii* Infected Golden Hamster

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Introduction

There are only a few reports concerning the antigenic components of *Toxoplasma gondii*. At least two precipitin lines were demonstrated by Strannegard (1962) by double diffusion-in-gel method in sera from patients suffering from toxoplasmosis and in sera from rabbits infected with *Toxoplasma*. Ouchterlony and immunoelectrophoretic analyses of the lyophilized *T. gondii* organisms obtained from the peritoneal exudate of white mice showed four components of parasitic origin (Chordi *et al.* 1964). The same methods were used by Szaflarski *et al.* (1966) when they reported the presence of a few precipitin lines from the supernate of frozed and thawed *Toxoplasma* from mice. Using golden hamsters as experimental host, Takayanagi *et al.* (1970) showed seven precipitin lines from homogenized *T. gondii* by the double gel diffusion in tube method of Preer (1956). The components were each named antigen a, b, c, d, e, f, and g, in their order from top to bottom of the tube.

Nonetheless, the presence of precipitating antigens in parasite-free exudate of infected mice has also been reported. Strannegard (1962) showed that the supernate of centrifuged exudate of infected mice contained as many precipitating antigens as did the homogenized sediment of the same exudate. Two parasitic components were shown by Chordi *et al.* (1964) in the supernate of centrifuged exudate. In an earlier report (Takayanagi *et al.*, 1970), the parasite-free exudate of infected golden hamsters was shown to contain two antigenic components corresponding to the c and d antigens of the homogenized *T. gondii*. The present report deals with the isolation and characterization of the antigens found in the parasite-free peritoneal exudate of infected golden hamsters.

Materials and Methods

Stock of *T. gondii* RH strain is maintained in the laboratory by serial transfer in mice every three days. For this study, golden hamsters were experimentally infected intraperitoneally with 0.5 ml of exudate from a previously infected mouse. Exudates were collected three days after infection by pipetting directly from the opened peritoneal cavity. Complete removal of the parasites was done by centrifugation of the material at 3,000 rpm for 15 min. The supernate was lyophilized and stored in powder form. The sedimented parasites were homogenized by means of an Umeda Oscillator, Sonore 150S at 200 mA for 5 min and centrifuged at 10,000 rpm for 30 min. The crude extract was stored at −23°C.

Antiserum was obtained from a rabbit inoculated with about 10,000 parasites intra-

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peritoneally and then treated with pyrimethamine at 20 mg/kg/day until cured. Blood was collected by cardiac puncture and the serum was inactivated at 56°C for 30 min. The method of Coons *et al.* (1955) was followed in the absorption of the serum with powdered hamster liver for 1 hr at 37°C. The absorbed serum was then stored at −23°C.

Agar gel diffusion tests were done by the modified methods of Ouchterlony (1958) and Preer (1956) using 1 % Difco Bactoagar in veronal buffer with an ionic strength of 0.05 at pH 8.6. With the Ouchterlony method, the distance between the antiserum well and the test antigen well was 5 mm. For Preer's test, pyrex tubes, 0.8 mm in internal diameter were used. Agar layer was 5 mm while both the antigen and the antibody layers were each 1 cm long.

Electrophoresis was carried out with cellulose acetate membranes immersed in veronal buffer at an ionic strength of 0.05 at pH 8.6. A direct current of 1 mA/cm for 3 hr at 4°C was applied. After electrophoresis, the membranes were dried and stained with 0.1 % Amido Black 10B in methanol-acetic acid solution (95 : 5) for about 5 min. Excess stain was washed off with methanol.

Fractionation with $(NH_4)_2SO_4$ was done as follows: To the parasite-free exudate, pH 7.0, solid $(NH_4)_2SO_4$ was added slowly to give 60 % saturation. After centrifugation at 5,000 rpm for 30 min, both the supernate and the sediment were dialyzed separately against phosphate buffer saline, pH 7.0, for at least three days with daily changes of the buffer. The entire process was done at 4°C. The materials were then lyophilized before analysis.

Column chromatography with DEAE-cellulose was carried out for both the supernate and the sediment. A column, 1 cm in diameter and 20 cm in length was used. Phosphate buffer (PB), pH 6.0, at concentrations 0.0175 M, 0.04 M, 0.10 M, and 0.40 M was used for stepwise elution. The eluted materials were each referred to as fraction I, II, III, and IV, respectively.

To determine the presence of acid-stable components, a small amount of the exudate was adjusted to pH 2.0 by dialyzing it overnight against 0.1 M KCl-HCl buffer. After dialysis, the material was centrifuged at 5,000 rpm for 5 min. The precipitate was discarded and the material was dialyzed against PB, pH 7.0. Ouchterlony method was used for analysis.

Results

The presence of antigens of parasitic origin in the parasite-free peritoneal exudate is shown in Figure 1. Two precipitin lines are shown, the upper one corresponding to antigen c and the lower one to antigen d as given in a previous report (Takayanagi *et al.*, 1970).

Fractionation of the exudate by means of $(NH_4)_2SO_4$ at 60 % saturation separated the two antigens from each other. The results of electrophoretic analysis of both fractions are shown in Figure 2. The supernate gave protein bands, one with a low mobility toward the negative side. Preer analysis revealed that the protein band on the negative side was derived from the parasite and corresponded to the c antigen. That on the positive side proved to be of host's origin, possibly albumin. The sediment, likewise, gave two protein bands, one that migrated to the negative side and the other, a diffused band that moved very slowly to the positive side. Preer analysis showed that both bands contained antigenic components of parasitic origin. The protein band on the negative side was found to be antigen b while that on the positive side consisted of gamma globulin from the host and the d antigen. Antigen b did not appear as a separate line with Preer analysis (Fig. 1) and was, therefore, not detected in an earlier study.

Column chromatography of the supernate gave an elution pattern shown in Figure 3. Two major peaks are observed, one at fraction I, and another at fraction III. Preer analysis (Fig. 4), however, showed *only one* precipitin line in fraction I and none in other fractions. The antigenic component in fraction I corresponded to antigen c.

Figure 5 shows the elution pattern of column chromatography made on the sediment. Shown are the protein peaks in all four fractions. Ouchterlony analysis (Figs. 6 and 7) showed two components of parasitic origin contained separately in fractions III and IV. Fraction III was found to contain antigen d, while fraction IV contained the b antigen.

Non-identity of the three identified antigens was established by Ouchterlony analysis (Figs. 8 and 9). Similar analysis of the unfractionated exudate (Fig. 10) showed that b and c formed overlapped precipitin lines when reacted with antiserum, hence the presence of only two lines, the outer line made up of b and c and the inner line, the d antigen.

When tested for the presence of acid-stable components, the unfractionated exudate at pH 2.0 was found to contain one, the c antigen (Fig. 11).

Discussion

Parasite-free peritoneal exudate of laboratory animals experimentally infected with *T. gondii* has been shown to contain precipitation antigens. The few available reports, however, do not completely agree on the number of such antigens which varies from two (Chordi *et al.* 1964; Takayanagi *et al.*, 1970) to as many as the homogenized material (Strannegard, 1962).

Strannegard (1962) showed that the parasite-free supernate of the exudate from infected albino mice contained as many precipitinogenic factors as did the *Toxoplasma*-rich sediment of the same exudate. He attributed this to two factors: (1) the possible disruption of the parasite during the process of centrifugation, and (2) the antigenic factors could be metabolic products or soluble toxins from the parasite. In this study conclusive evidence points to the presence of at least three antigens of parasitic origin in the peritoneal exudate. The various methods employed in the analysis suggest that all antigens are metabolic products of the parasite activities, and are, therefore, called soluble antigens or exoantigens. As has been mentioned, a total of seven antigenic components was found in the homogenized *T. gondii* by means of the Preer analysis. Of these seven, three components, b, c, and d, have been consistently found in the parasite-free exudate of infected golden hamsters while the other four components were never seen to occur with the various tests used. From these results, the presence of the antigens in the exudate may not have been caused by the mechanical disruption of the parasites. In other words, these antigens are products of parasite metabolism. The presence of more than one exoantigens has been found also in *Trichomonas foetus* (unpublished report).

Separation of these three components have been done satisfactorily as shown by the results of the different methods used. Further analysis and purification of the isolated antigens are presently being undertaken.

The role each of these identified exoantigens play in the course of infection is not known as yet. It is not difficult to assume, however, that these antigens make possible the continuous existence and multiplication of the parasite in the body of the host despite the presence of antibodies formed against it. Matsubayashi and Akao (1966) report that the intracellular parasites are protected from the effects of the antibody by a barrier posed by the limiting membrane of the vacuole within which the parasites are located. They have indicated the presence of high concentrations of antigens on this membrane which react with the antibody and then leave the parasite free to continue multiplication inside the cyst. The exoantigens herein reported may be the same antigens that protect the parasites by combining with antibody.

Summary

Parasite-free peritoneal exudate of golden hamsters infected with *Toxoplasma gondii* RH strain was analyzed for antigenic components of parasitic origin by fractionation

with $(NH_4)_2SO_4$ at 60 % saturation, electrophoresis, DEAE-cellulose column chromatography, and the gel diffusion tests of Preer and Ouchterlony. Three precipitating antigens, b, c, and d, were found and characterized. $(NH_4)_2SO_4$ fractionation sedimented antigens b and d, while c remained in the supernate. Both c and b showed electrophoretic mobility toward the negative side. Antigen d showed a very low electrophoretic mobility toward the positive side. Column chromatography separated b and d from each other; antigen b eluted at 0.4 M phosphate buffer, pH 6.0, and antigen d at 0.1 M. In addition, antigen c was found to be acid-stable.

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Toxoplasma gondii 感染ハムスターの腹水中にみられる遊離抗原の分析

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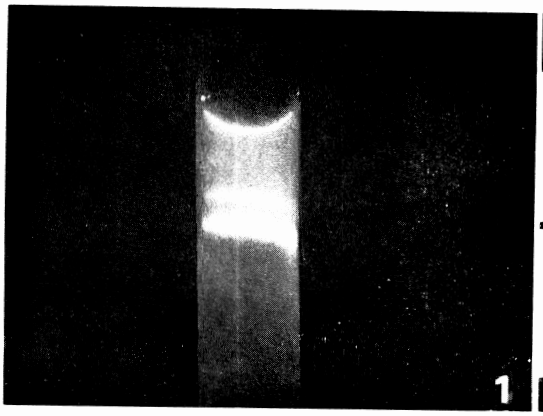
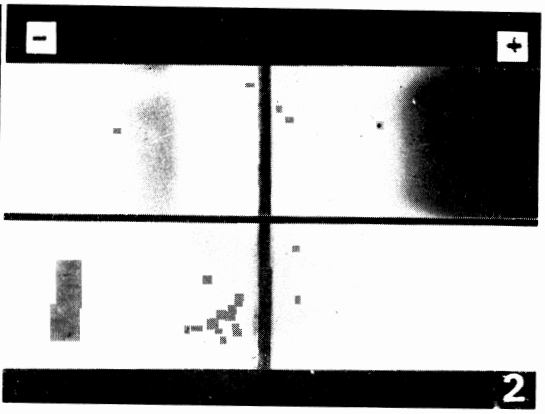
トキソプラズマ RH 株を接種したハムスターの腹水を遠心して原虫を除去し，遠心上清にみられる原虫由来の抗原について硫安分画，電気泳動，DEAE セルローズカラムクロマトグラフィーおよび寒天ゲル内沈降反応（Preer および Ouchterlony 法）により分析した．

この腹水中には3種類の抗原 b, c および d (Takayanagi *et al.*, 1970) が認められた．硫安 60 %飽和では抗原 b と d が沈殿中にみられ抗原 c は上清に認められた．c および b は電気泳動により陰極側に泳動したが d は陽極側に泳動した．DEAE セルローズによる分画では b と d を互に分離することが出来，b は 0.4 M 燐酸緩衝液（pH 6.0）で流出し d は 0.1 M で流出した．c は b および d と異なり酸に安定であつた．

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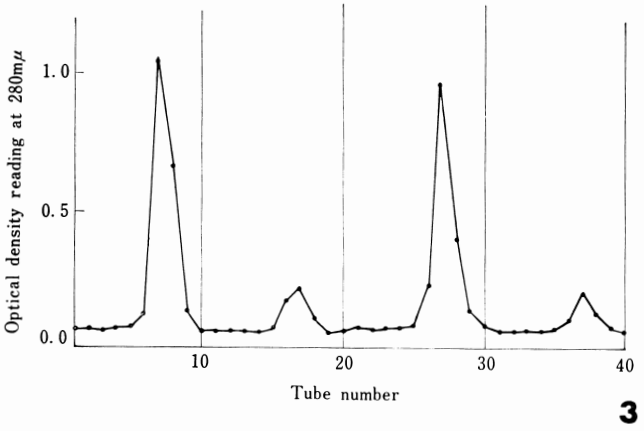
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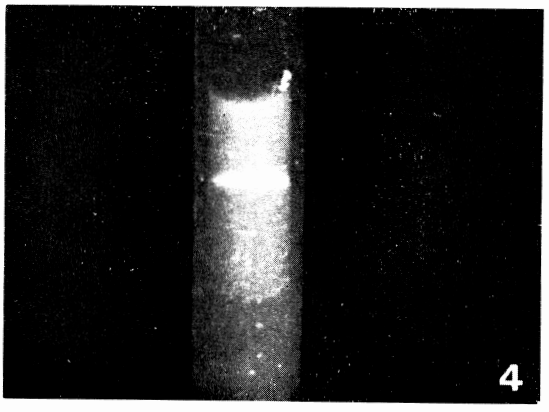

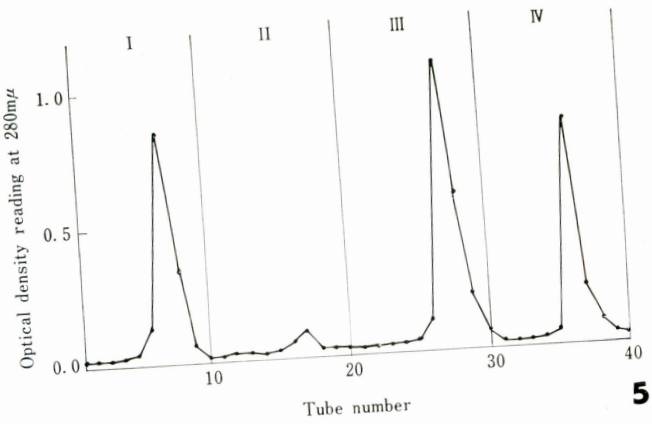
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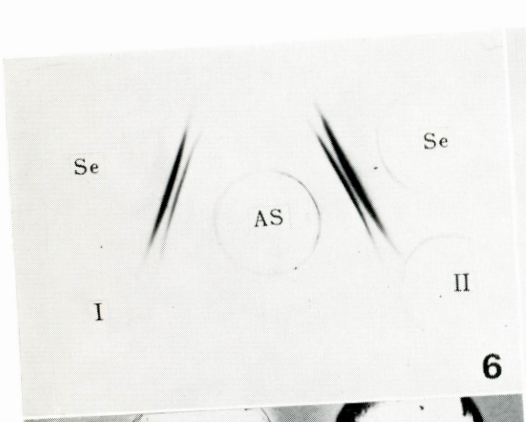
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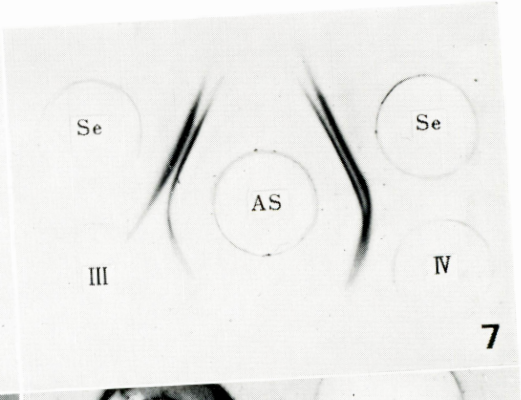
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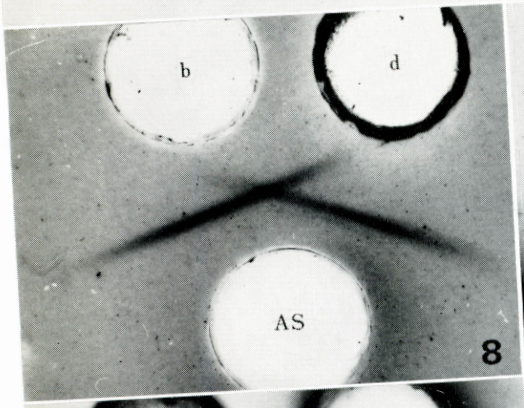
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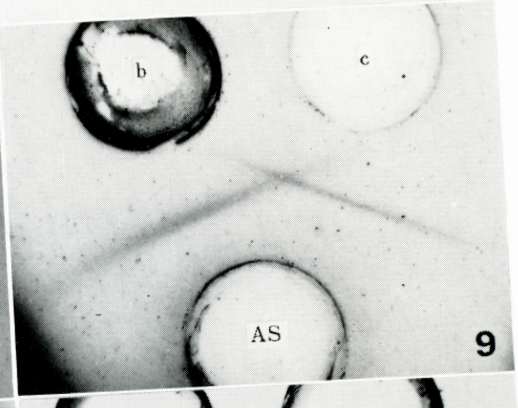
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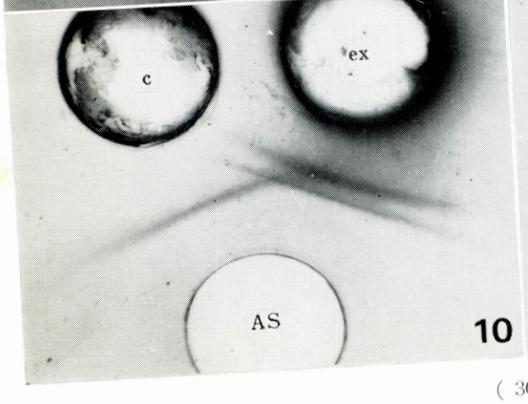
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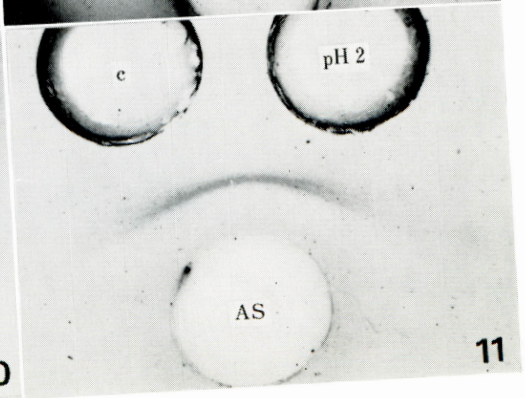
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Explanation of Figures

Figure 1. Preer analysis of the unfractionated, parasite-free exudate. Two precipitin lines are shown.

Figure 2. Electrophoretic patterns of the exudate after $(NH_4)_2SO_4$ fractionation. The supernate (upper slide) shows two bands that migrated separately. One antigenic component of parasitic origin was found contained in the band on the negative side. The sediment (lower slide) shows one distinct band on the negative side and another diffused band that migrated very slowly to the positive side. Both bands each contained one antigenic component of parasitic origin.

Figure 3. Elution pattern of the supernate on DEAE-cellulose column chromatography with phosphate buffer, pH 6.0 at concentrations 0.0175 M (I) ; 0.04 M (II) ; 0.10 M (III) ; and 0.40 M (IV). One antigenic component of parasitic origin was found contained in fraction I.

Figure 4. Preer analysis of fraction I of the supernate. The precipitin line is the c antigen.

Figure 5. Elution pattern of the sediment on DEAE-cellulose column chromatography with phosphate buffer, pH 6.0, at concentrations 0.0175 M (I) ; 0.04 M (II) ; 0.10 M (III) ; and 0.40 M (IV). Two antigenic components of parasitic origin were found each in fractions III and IV.

Figures 6 and 7. Ouchterlony analysis of fractions I, II, III, and IV of the sediment. Fraction III shows the isolated antigen d, while fraction IV contains mainly the b antigen. Se=sediment ; AS=antiserum.

Figures 8 and 9. Non-identity reactions among the identified and isolated antigens, b, c, and d.

Figure 10. Ouchterlony analysis of the unfractionated exudate (ex) and the antigenic component of the supernate (c). Antigens b and d are separated from each other while antigen c overlaps with the b antigen.

Figure 11. Ouchterlony analysis of the unfractionated exudate at pH 2.0. The acid-stable component is shown to be the c antigen.