

Antibodies of *Toxoplasma gondii**

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INTRODUCTION

In the diagnosis of toxoplasma infection it is frequently impossible to attempt isolation of the organism. Resort has to be made to the examination of serum. Numerous tests have been devised. The multiplicity of such tests suggest that difficulties regarding technique or interpretation are numerous. Although the dye test and the complement-fixation test are widely used, a haemagglutination test (Jacobs & Lunde, 1957), a direct agglutination test (Fulton & Turk, 1959), a flocculation test (Siim & Lind, 1960), an agar gel diffusion (O'Connor, 1957), and a fluorescent antibody test (Goldman, 1957) have all been offered as substitutes.

The work described here is concerned with certain difficulties in performing the dye test, and with evidence that there are two antigens of *Toxoplasma gondii* capable of fixing complement. A relationship between some of the tests is suggested.

THE DYE TEST

Sabin & Feldman (1948) while looking for an *in vitro* manifestation of neutralizing antibody found that toxoplasma subjected to antiserum in the presence of fresh normal human serum lost the capacity to stain with methylene blue.

Lelong & Desmots (1952) likened this effect to the Pfeiffer phenomenon. Toxoplasma antibody in the presence of fresh normal human serum produced a clarification of the normally granular cytoplasm of toxoplasma organisms.

Beverley & Beattie (1952) showed that the number of toxoplasma organisms in the reaction mixture modified the result of the dye test. They found that exudate removed from the mouse peritoneum more than 3 days after infection showed a high proportion of unstained organisms in the control tubes containing normal human serum but no added antibody. They considered this effect was due to antibody production in the mouse.

THE ANTIGENS OF *TOXOPLASMA GONDII*

Warren & Russ (1948), in developing the complement-fixation test for toxoplasma antibody, centrifuged the antigen they made at high speed in order to remove anticomplementary material. The complement-fixing antibody found by using this centrifuged antigen was less sensitive than the dye test.

Thalhammer (1960) used an uncentrifuged freeze-dried antigen, and obtained complement-fixing antibody titres similar to those of the dye test. It seems likely

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therefore that there are two antigens both capable of producing complement-fixing antibody. It is known that at least two toxoplasma antigens exist according to the experiments of Siim & Lind (1960) and Fleck (1961).

Huldt (1958), quoting an observation by Gard, states that there is a difference between the antigens responsible for the dye test and those responsible for the complement-fixation test. Gard's observation was that dye test antibodies could not be fully neutralized with complement-fixing antigen that had been prepared by centrifugation at high speed.

Cutchins & Warren (1956) showed that complement-fixing and dye test antibody followed the inoculation into rabbits of antigen centrifuged at high speed.

PRESENT INVESTIGATION

Four experiments were performed.

(A) To confirm that the number of toxoplasma organisms in the dye test affects the antibody titre.

(B) To attempt removal by absorption of the cytoplasm-modifying substance which occurs in the peritoneal exudate of mice injected 4 or more days previously with a large number of toxoplasma organisms.

(C) To try to imitate the dye test by using detergents or other chemicals in place of antibody.

(D) To note the effect of high-speed centrifugation on toxoplasma extracts used in the complement-fixation test, and the effect these extracts have, when mixed with toxoplasma antibody in the dye test.

EFFECT OF NUMBERS OF TOXOPLASMA ORGANISMS IN THE DYE TEST

Materials and methods

Dye tests were made, using peritoneal exudate from mice inoculated 3 days previously with 0.5 ml. of a toxoplasma suspension containing 1×10^6 parasites. Serial twofold dilutions of the resultant exudates were made in saline and titrated against a standard toxoplasma antiserum. Counts of the numbers of toxoplasma organisms in each test were made with a Neubauer counting chamber.

Comparative tests with a toxoplasma suspension grown in calf-kidney tissue culture were performed. The suspensions were made by inoculating a bottle of calf-kidney tissue culture containing 5×10^6 cells with approximately 5×10^6 toxoplasma organisms. After 2 days most of the calf-kidney cells had liberated large numbers of toxoplasma organisms. These were washed twice with growth medium consisting of Hank's balanced salt solution with the addition of 0.4% lactalbumen, 0.051% sodium bicarbonate and 20% bovine serum. Antibiotics were added to prevent contaminants growing in the medium. These included penicillin, streptomycin and nystatin at a concentration of 100 units/ml. together with neomycin at a concentration of 50 units/ml.

The toxoplasma organisms were resuspended in saline and counted. Serial twofold dilutions were made and titrated with standard antiserum as with the mouse exudates.

Accessory factor was obtained from normal human blood donors and consisted of serum removed from clotted blood less than 48 hr. before being stored at -20°C . The serum was tested and shown to be free from toxoplasma antibody. No deterioration in serum stored for as long as 10 months was noted.

Results

Table 1 shows the effect of the number of organisms on the standard serum titre. The higher the number of organisms in the reacting mixture the lower the dilution at which the antiserum 50% end-point occurs. This effect still persisted when the parasites were washed with normal fresh human serum, and when suspensions of organisms were prepared from tissue cultures. There was however considerable variation in the titres obtained with unwashed, washed and tissue culture antigens.

Table 1. *Effect of number of toxoplasma organisms on the dye test carried out with a standard antiserum*

Dye tests with unwashed toxoplasma from mouse peritoneum			Dye tests with washed toxoplasma from mouse peritoneum			Dye tests with washed toxoplasma from tissue culture		
Antigen dilutions	No. of orgs./ml.		Antigen dilutions	No. of orgs./ml.		Antigen dilutions	No. of orgs./ml.	
	Antiserum (50% end-points)	in millions in saline control tube		Antiserum (50% end-points)	in millions in saline control tube		Antiserum (50% end-points)	in millions in saline control tube
Neat	1/80	8.6	Neat	1/40	10	Neat	1/160	35
1/2	1/640	3.0	1/2	1/160	6.4	1/2	1/640	16
1/4	1/1280	1.9	1/4	1/320	2.9	1/4	1/640	8
1/8	1/1280	1.2	1/8	1/320	1.8	1/8	1/640	4

EFFECT OF MOUSE ANTIBODY IN MOUSE EXUDATE ON THE DYE TEST

Peritoneal exudate of mice infected with 1×10^6 toxoplasma organisms was only usable in the dye test when removed from the mouse 3 days after inoculation. If the exudates were removed from the mice 4 or more days after inoculation they showed poor staining when placed in control tubes containing accessory factor and saline only. When supernatant fluid from the centrifuged exudate of a mouse inoculated 4 days previously was absorbed overnight with a washed toxoplasma suspension at room temperature (22°C .), the supernatant did not interfere with the staining of a toxoplasma suspension. The substance appearing in the peritoneal exudate of a mouse injected 4 days previously with toxoplasma and which interferes with the dye test is precipitated with the gamma globulin protein fraction and withstands heating for 1 hr. at 56°C . It is therefore highly likely to be toxoplasma antibody and not a non-specific anti-toxoplasma factor.

EFFECT OF SURFACE ACTIVE AGENTS ON TOXOPLASMA

Lelong & Desmonts (1952) likened the effect of antibody on toxoplasma to the Pfeiffer phenomenon. They considered that antibody lysed the parasites producing clarification of the cytoplasm. The present author thought that other substances might produce lysis of the organism and imitate the dye test.

Materials and methods

Serial twofold dilutions of various chemicals, mostly detergents, were made in physiological saline. A dye test was carried out with these dilutions instead of with test serum. One volume of diluted chemical, one volume of toxoplasma exudate and two volumes of accessory factor were mixed, and incubated in a water-bath for 1 hr. The mixtures were then stained with alkaline methylene blue for 5 min. and the parasites examined by a wet film preparation. The approximate pH of each chemical solution was measured by making a 10% solution of B.D.H. Universal Indicator in the chemical being examined.

Table 2. *Effect of various chemicals on the staining capacity of Toxoplasma gondii*

Chemical	Effect	pH of solution
Sodium lauryl sulphate	Modification at dilution 1/2	6.0
Sodium stearate	No modification with saturated solution	9.0
Teepol	Modification at dilution 1/10	7.0
Domiphen bromide	No modification found	6.5
Cetyl trimethyl ammonium bromide	Modification at dilution 1/32	7.0
Tween 80	Modification at dilution 1/4	6.0
Sorbitan mono-oleate	No modification with saturated solution	6.5
Sodium taurocholate	Modification at dilution 1/10	5.0
Sodium deoxycholate	Modification at dilution 1/10	7.0
Lysol	Lysis but no modification at dilution 1/16 or less	4.0
Phenol	Lysis but no modification at dilution 1/2 or less	4.0
Sodium azide	Modification at dilution 1/2	11.0
Sodium chloride	No modification with saturated solution	7.0

Results

Table 2 shows the effect of a range of detergents on a suspension of toxoplasma in saline containing accessory factor but no antibody. Some of the detergents and other chemicals modified the staining of the parasites so that the appearance resembled a positive result in the dye test. This effect only occurred however when the chemicals were used in very high concentrations. No correlation could be found between the type of detergent (i.e. anionic, cationic or non-ionic) and its effect.

Although such a non-specific effect is unlikely to occur in practice, dye tests should be set up with controls containing serum to be tested and toxoplasma suspension only.

THE EFFECT OF HIGH-SPEED CENTRIFUGATION ON TOXOPLASMA EXTRACTS

Complement-fixation tests

Peritoneal exudate from mice inoculated 3 days previously with a suspension of live toxoplasma was washed in physiological saline centrifuged at 3000 r.p.m. for 10 min. The deposit was then mixed with 10 times its weight of distilled water and allowed to lyse at 4° C. overnight with occasional shaking. The mixture was then frozen and thawed 4 times and centrifuged at 3000 r.p.m. for 10 min. to remove solid material. An equal volume of double strength saline was added to restore isotonicity; this mixture will be described as whole-body extract. Some of this whole-body extract was centrifuged at about 25,000 g. and the supernatant designated the light extract. Early experiments showed that optimum antigen dilutions were difficult to demonstrate, the antigen concentrations being too weak. The antigen solutions were then concentrated to 1/10 of their original volume by placing a cellophane dialysis sac packed with polyethylene glycol (carbowax; 20M) in the antigen solution.

Complement-fixation tests with serial twofold dilutions of extract, against fourfold dilutions of a control antiserum were made in chess board patterns on plastic plates. 2 M.H.D. of guinea pig complement was used with overnight fixation at +4° C. After fixation 2% sheep cells sensitized with 5 M.H.D. of H.I.B. were added and the mixtures incubated for 30 min. at 37° C. The mixtures were stored at 4° C. for 3 hours and the results read.

Table 3. *Complement-fixation tests (concentrated toxoplasma extracts × standard toxoplasma antiserum)*

	Extract dilutions									Control (no antigen)
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	
Extract was heated and centrifuged at high speed	AC	<u>256</u>	<u>64</u>	<u>64</u>	<u>64</u>	<u>64</u>	32	32	32	0
Extract was unheated ; centrifuged at high speed	AC	AC	AC	4000	256	<u>64</u>	<u>64</u>	<u>64</u>	<u>64</u>	0
Extract was heated but ; centrifuged at high speed	AC	<u>1000</u>	<u>1000</u>	<u>1000</u>	<u>1000</u>	<u>1000</u>	<u>1000</u>	128	64	0
Extract was not heated and not centrifuged at high speed	AC	AC	AC	AC	4000	1000	256	64	64	0

Figures indicate reciprocals of titres of standard antitoxoplasma serum showing 50% haemolysis. C = Anticomplementary. Titres underlined are those showing a plateau of constant antibody with varying antigen concentration.

Dye-test inhibition

Concentrated extracts prepared in various ways were added to equal volumes of high titre human antisera for 1 hr. at 20° C. These mixtures were then added to the dye test and the antibody content found.

Any inhibition of antibody by the extract was noted by the fall in antibody titre. A control mixture containing saline and antibody in equal amounts was set up.

Results

Table 3 shows the results of the complement-fixation tests with extracts from infected mouse peritoneal exudates. Each experiment shows the same batch of extract treated in four different ways, i.e. heated at 56° C. for 1 hr. and centrifuged at high speed; unheated and centrifuged at high speed; heated but not centrifuged; and unheated but not centrifuged.

It can be seen that heating to 56° C. for 1 hr. removes a large proportion of the anticomplementary material.

Centrifugation produces a pattern with 50% complement fixation by antibody and extract extending over a range of three dilutions at a serum titre of 1/64. Uncentrifuged antigen produces a pattern with 50% fixation extending over a wide range at a titre of 1/1024. Heating produced only a slight fall in the sensitivity of the extract.

This suggests that there are two complement-fixing antigens of different densities. The whole body extract used as antigen gives complement-fixation titres which correlate quite closely with dye test, but the lighter extract gives complement-fixation titres much lower than those of the dye test.

Table 4 shows that mixing the light extract with antiserum and performing a dye test on the mixture does not reveal antibody inhibition. Mixing the whole body extract with antiserum and performing a dye test on the mixture does show incomplete dye-test antibody inhibition.

Table 4. *Effect of concentrated toxoplasma extracts on the dye test*

Toxoplasma extracts (concentrated with carbowax)	Standard serum dilution giving 50% end-point in dye test
(1) Heated to 56° C. for $\frac{1}{2}$ hr. and centrifuged at 25,000g	1/2000
(2) Heated to 56° C. for $\frac{1}{2}$ hr. but centrifuged at 1500g only	1/32
(3) Unheated but centrifuged at 25,000g	1/2000
(4) Unheated but centrifuged at 1500g only	1/16

Dye tests were performed on mixtures of equal volumes of supernatants from toxoplasma extracts and antiserum.

Control tests, containing all four forms of toxoplasma extracts without accessory factor or antiserum, were negative in the dye test.

Controls containing accessory factor and saline were also negative.

DISCUSSION

In an effort to find a simple, safe, economical substitute for the dye test, many tests for toxoplasma antibody have been devised. Many of these have produced results which, in the hands of some workers, have correlated well with the dye test, but others have shown the correlation to be poor. Whether these variations are due to differences in technique or to the measurement of different antibodies is difficult to say.

Beverley & Beattie (1952) demonstrated that the number of toxoplasma organisms in the dye test affects the end-point of the serum being measured.

That this effect is not due to soluble antigen is shown by washing the parasites and resuspending them in accessory factor. After this treatment the effect still persists.

The specificity of the dye test has frequently been impugned. Mas-Bakal (1959), however, showed that the test did not become positive in serum after the inoculation of various bacterial vaccines, viruses or protozoa into experimental animals. Kulasiri (1960) could incriminate no protozoal parasite, except toxoplasma, in the production of dye-test antibody. Feldman (1956) however demonstrated a non-specific thermolabile toxoplasma-modifying effect in animal sera. While looking for some substance which would produce a non-specific result in the dye test the author has found that many substances in high concentrations would modify the staining capacity of toxoplasma.

There is little doubt that there are at least two antigens contained in *Toxoplasma gondii*. These are demonstrated by the different antibodies they produce. It has been shown that the antigen and antibody responsible for the dye test are different from those of the haemagglutination test (Fleck, 1961). Siim & Lind (1960) stated that preliminary results of absorption experiments show evidence of existence of more than one antigen.

Warren & Sabin (1942) first demonstrated complement-fixing antigen in a rabbit brain suspension of toxoplasma. Warren & Russ (1948) produced toxoplasma antigen from infected chorioallantoic membrane; they removed the anticomplementary effect by centrifugation at 14,000 r.p.m. for 1 hr. Sabin (1949) showed that high-speed centrifugation was less likely to produce an antigen which fixed complement non-specifically in the presence of serum from patients immunized with egg vaccines. Toxoplasma antibody measured with this centrifuged complement-fixing antigen appeared later in the disease than the dye test antibody and disappeared earlier. Steen & Kass (1951) produced an antigen by lysing a toxoplasma suspension with water. This antigen was specific and was not anti-complementary. It had not been centrifuged at high speed. Thalhammer (1956) found that this antigen gave results which agreed very well with the dye test.

The present work shows that the serum titre showing 50% haemolysis in the complement-fixation test over a wide range of antigen titres, with an antigen centrifuged at high speed, differs from that shown by an antigen centrifuged at low speed. The latter material must contain a light and heavy component. The material centrifuged at high speed must contain the light component only. This light antigen was unable to inhibit the antibody responsible for the dye test.

It is suggested therefore that the soluble antigen remaining after high-speed centrifugation be called the light antigen, that the antigen which is removed by centrifugation be called the heavy antigen. Further work is needed to show the relationship of these two antigens to other tests. It may be that the heavy antigen is responsible for the dye-test antibody as well as the complement-fixation antibody described by Thalhammer (1956) and by Steen & Kass (1951). Tonjum (1962) has shown that two lines are produced when the supernatant from the peritoneal exudate of a toxoplasma-infected mouse is allowed to diffuse against immune serum in agar. Agar gel diffusion tests performed in this laboratory suggest that at least one of the lines is due to the light antigen. Preliminary work suggests that

antibody responsible for the direct agglutination test of Fulton & Turk (1959) is related to the antibody responsible for the dye test rather than that for the haemagglutination test.

SUMMARY

1. That the number of toxoplasma organisms in the dye test affects the titre of the test serum is confirmed.

2. Evidence is presented that the toxoplasma cytoplasm-modifying substance present in the peritoneal exudate of mice injected with large numbers of toxoplasma organisms more than 3 days previously is probably antibody.

3. Various chemicals including detergents were capable, in high concentration, of producing a false positive dye test.

4. Evidence is presented that there are two complement-fixing antigens, one with a low sensitivity and a low density, the other with a high density and a sensitivity close to that of the dye test. Heating the heavy antigen to 56° C. for 1 hr. removes most of its anticomplementary effects without altering its complement-fixing activity.

5. The light antigen mentioned above was capable of producing a precipitation line in agar double-diffusion experiments, but did not inhibit dye-test antibody when mixed with an equal volume of antiserum in the dye test.

6. The heavy antigen inhibited dye test antibody when mixed with an equal volume of antiserum in the dye test.

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REFERENCES

- BEVERLEY, J. K. A. & BEATTIE, C. P. (1952). *J. clin. Path.* **5**, 350.
 CUTCHINS, E. C. & WARREN, J. (1956). *Amer. J. trop. Med.* **5**, 197.
 FELDMAN, H. A. (1956). *Ann. N.Y. Acad. Sci.* **66**, 263.
 FLECK, D. G. (1961). *Nature, Lond.*, **190**, 1018.
 FULTON, J. D. & TURK, J. L. (1959). *Lancet*, ii, 1068.
 GOLDMAN, M. (1957). *J. exp. Med.* **105**, 557.
 HULDT, G. (1958). *Acta path. microbiol. scand.* **43**, 141.
 JACOBS, L. & LUNDE, M. N. (1957). *J. Parasit.* **43**, 308.
 KULASIRI, C. (1960). *J. clin. Path.* **13**, 339.
 LELONG, M. & DESMONTS, G. (1952). *C.R. Soc. Biol., Paris*, **146**, 207.
 MAS-BAKAL, P. M. (quoted by A. POLMAN) (1959). *Trop. Geograph. Med.* **11**, 13.
 O'CONNOR, G. R. (1957). *Arch. Ophthalm., Chicago*, **57**, 52.
 SABIN, A. B. (1949). *Paediatrics*, **4**, 443.
 SABIN, A. B. & FELDMAN, H. A. (1948). *Science*, **108**, 660.
 SIM, J. C. & LIND, K. (1960). *Acta path. microbiol. scand.* **50**, 445.
 STEEN, E. & KASS, E. (1951). *Acta path. microbiol. scand.* **28**, 36.
 THALHAMMER, O. (1956). *Mtschr. Kinderheilk.* **104**, 110.
 THALHAMMER, O. (1960). *Human Toxoplasmosis*, p. 196. Copenhagen: Munksgaard.
 TONJUM, A. M. (1962). *Acta path. microbiol. scand.* **54**, 96.
 WARREN, J. & RUSS, S. B. (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 85.
 WARREN, J. & SABIN, A. B. (1942). *Proc. Soc. exp. Biol., N.Y.*, **51**, 11.