THE LABORATORY DIAGNOSIS OF TOXOPLASMOSIS

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A case of congenital toxoplasmosis has recently been seen in this hospital. The patient, a girl aged 5 years, presented with hydrocephalus, bilateral pes cavus, and blindness. The eyes showed extensive retinochoroiditis thought to be characteristic of toxoplasmosis. Radiographs of the skull showed areas of cerebral calcification. Two years before the birth of the patient the mother had suffered from an illness accompanied by a rash and headache, thought to be rubella. Otherwise there was no family history suggestive of toxoplasmosis, and the mother was in good health. The clinical aspects of this case are being reported elsewhere.

Although cases of toxoplasmosis are being reported from all over the world, so far only three cases have been published in England. In the case of Jacoby and Sagorin (1948) the serological confirmation of the diagnosis was obtained from Dr. Sven Gard, of Stockholm, and in the two cases of Farquhar and Turner (1949) the serology was done by Dr. Sabin, of Cincinnati. Owing to the apparent rarity of the disease few laboratories in this country deemed it worth while maintaining a strain of *Toxoplasma*, but as more cases are suspected on clinical grounds laboratory methods of investigation will have to be made available. This communication is an account of the diagnostic laboratory tests at present in use and our experiences with them in the case mentioned above.

The laboratory methods available for the diagnosis of toxoplasmosis are the isolation of the organism and the demonstration of antibodies to *Toxoplasma* in the patient's serum. In addition, a skin reaction, similar to the tuberculin reaction, may be obtained in positive cases with an antigen prepared from the *Toxoplasma*. All these tests are associated with the names of Sabin and his associates, on whose writings we have freely drawn. The clinical pathology of the condition affords little diagnostic help, although in the acute phase an increase in cells and protein in the

cerebrospinal fluid is sometimes seen (Sabin, 1942a), an increase in protein but not in cells being occasionally found in apparently healed cases. No specific blood changes or eosinophilia have been reported. In the present case both the cerebrospinal fluid and the blood picture were normal.

Attempts to Isolate Toxoplasma

A centrifuged deposit of the cerebrospinal fluid was examined microscopically for *Toxoplasma*, with negative results. Part of the deposit was inoculated intraperitoneally and intracerebrally into mice. After one month, during which the mice remained well, they were sacrificed and emulsions of their brains and viscera were inoculated into further mice. This precautionary passage is necessary because sometimes primary inoculation of infected material causes no manifest disease, whereas material from such mice is capable of causing sickness when passaged to further mice.

In this experiment none of the mice became ill at any stage. In view of the long clinical history of our case and the normal cerebrospinal fluid this negative result was not surprising.

As *Toxoplasma* is apparently an obligate intracellular parasite, multiplying within fixed tissue cells, it is unlikely that an artificial culture medium will be elaborated for its isolation. In the present case, cultures were attempted from the cerebrospinal fluid on several occasions into Boeck and Drbohlav's, N.N.N., and Harding and Hawking's (1944) trypanosome medium, with negative results. Inoculation into developing eggs was also unsuccessful.

Serological Methods

So far, neutralizing (Sabin and Olitzky, 1937), complement-fixing (Warren and Sabin, 1942), and cytoplasm-modifying (Sabin and Feldman, 1948) antibodies to *Toxoplasma* have been described. The complement-fixing antibody appears to be different from the neutralizing antibody in that it is more heat stable and may appear later and

disappear earlier in the disease. Similarly, it is different from the cytoplasm-modifying antibody in that sera containing a high titre of the latter may be devoid of complement-fixing antibody. Cytoplasm-modifying and neutralizing antibodies usually seem to occur together, but whether they are in fact identical is not yet clear. For these reasons we deal with the three antibodies and their demonstration separately, despite the fact that what is called the cytoplasm-modifying antibody may be the neutralizing antibody reacting in a sphere other than the rabbit skin.

Sabin (1948) expresses dissatisfaction with the rabbit neutralization test on the ground that, while it is capable of demonstrating the presence of neutralizing antibodies in the serum under investigation, it is impossible to tell whether these were acquired as the result of the present clinical disease or as a result of previous inapparent infection. He also states that these antibodies may not be present shortly after infection has taken place or some years after the infection has burned out, so that a negative complement-fixation test may be misleading. The cytoplasm-modifying antibody test and the quantitative data yielded by it he regards as most valuable in deciding the state of activity in a given case.

The Rh strain of *Toxoplasma*, a strain virulent for mice, was obtained through the courtesy of Colonel H. E. Shortt, of the London School of Hygiene and Tropical Medicine. This is the strain originally isolated by Sabin in 1939 from a case of acute encephalitis (Sabin, 1948), and it has since been maintained by passage in mice. Blood from the patient and her mother was withdrawn by venepuncture and allowed to clot in the refrigerator. The serum was separated four hours later and divided into 1-ml. amounts. Half of these were immediately frozen solid and stored at -60°C ., while the remainder were inactivated at 60°C . for 20 minutes for use in complement-fixation reactions and thereafter stored at -20°C .

Neutralizing Antibody. — Levaditi, Sanchis-Bayarri, Lépine, and Schoen (1929), working with *Toxoplasma* infection in rabbits, were unable to demonstrate any neutralizing antibody in convalescent serum, and concluded that the resulting immunity, which was absolute, was entirely cellular in origin. Sabin and Olitzky (1937), however, showed that there was a species specificity to toxoplasmosis. The majority of smaller animals, such as mice, guinea-pigs, and rabbits, died of the infection, and survivors did not show any demonstrable antibody. Rhesus monkeys, on the other hand, developed a non-fatal infection, and their

convalescent serum had the power to neutralize *Toxoplasma* when injected intracutaneously into the skin of a rabbit. Sabin (1941) showed that this neutralizing antibody also developed in human beings, and described a convenient method for its titration using the intracutaneous route in the rabbit. He also showed that the neutralizing antibody had no apparent *in vitro* effect on the *Toxoplasma*, as no agglutination or lysis could be detected in mixtures of immune serum and *Toxoplasma* suspensions. Sabin and Ruchman (1942), working with rhesus monkeys, showed that this neutralizing antibody had certain unusual characteristics. It appeared early in the disease and persisted for at least 15 months without any marked change in titre. They also showed that the antibody was of low titre and extremely labile. It was inactivated at 56°C . for 30 minutes and the titre dropped markedly if kept at room temperature or stored at 4°C . for a few days. They found that the only method of preserving the antibody was by freeze-drying or by keeping the serum at -60°C . (This factor is of great importance when testing for neutralizing antibodies in the laboratory.) Sabin (1942a) showed that the rabbit skin neutralization test was a reliable method for the diagnosis of the disease in humans, and that a significant number of patients in whom the disease was suspected clinically had demonstrable neutralizing antibodies in their serum. Serum from the mothers of these cases usually had neutralizing antibodies as well.

Heidelman (1945) obtained a weak to strongly positive result in neutralizing antibody tests in 63% of a series of cases presenting congenital retinochoroiditis, and in six out of seven mothers of infants with congenital retinopathy. Sabin (1942b), in a group of 151 selected individuals, children with nervous and ocular diseases and many mothers of such children, found neutralizing antibodies in the sera of 59. Also, of 15 children presumably infected *in utero* with *Toxoplasma*, he observed that 13 of the mothers gave a positive neutralizing antibody test. Similarly Johnson *et al.* (1946), reviewing presumptive cases of toxoplasmosis from the literature and their own centre, reported that of 45 patients with retinochoroiditis and serum neutralizing antibodies only seven failed to show radiological evidence of cerebral calcification. Further, taking 32 selected patients of various ages with central retinochoroiditis, active or inactive, they found that 20 possessed neutralizing antibodies in their sera. Crothers (1943) recorded nine children, belonging to five families, in which a clinical diagnosis of toxoplasmosis was

supported by serological evidence. All the children had cerebral calcification, and seven of them had inactive retinochoroiditis. The disease affected two children in two of the families, and the mother of one of the pairs had cerebral calcification. The presence of antibody in the sera of healthy siblings is common. In a study of a particular family with a toxoplasmic member Johnson *et al.* (1946) found neutralizing antibodies in seven out of a total of nine children.

In normal people and others with acquired retinochoroiditis Heidelman (1945) found evidence of neutralizing antibody in the sera of 10%. Choosing 58 normal people at random Binkhorst (1948) found antibody in the sera of six. Johnson *et al.* stated that four laboratory workers dealing with *Toxoplasma* research altered from negative to positive in their serum reactions, but no sign of illness occurred.

Rabbit Skin Method.—This test was carried out in the present cases by the method suggested by Sabin and Ruchman (1942). Serial tenfold dilutions of *Toxoplasma*-infected mouse brain in Tyrode's solution were mixed with equal quantities of undiluted test serum. These were allowed to stand at room temperature for 30 minutes. Two controls were used, one incorporating a negative serum, the other in which serum was replaced by Tyrode's solution. After standing, 0.2 ml. was injected intracutaneously into a rabbit's back which had been suitably prepared for inoculation. Erythema usually developed around the serum-*Toxoplasma* mixtures after 24 hours and persisted for two to three days. Erythema and induration going on to necrosis developed on the third to fourth day, being maximal in those areas with higher concentrations of *Toxoplasma*. Readings were taken on the fifth and eighth days. The rabbits usually died between the tenth and twelfth

days of generalized toxoplasmosis. The results of the test are shown in Table I.

Difficulty was experienced in repeating these results. Accordingly, several variations in technique were tried, and we found that *Toxoplasma*-infected mouse peritoneal fluid gave results which could be reproduced more accurately.

This test is undoubtedly of great value in diagnosis but presents many technical difficulties, and when only a few sera are being tested at a time and in the absence of a known positive control the interpretation of the results is made even more difficult. Professor C. P. Beattie, of the Department of Bacteriology, University of Sheffield, very kindly undertook to check these sera for us. He found that V.D.'s serum neutralized 100 rabbit skin doses, whereas Mrs. D.'s serum gave an indeterminate result.

Chick Embryo Method.—Using the technique of Warren and Russ (1948) we adapted the Rh strain to the chorio-allantoic membrane. This appeared to offer several advantages. First, it avoided the necessity for continual animal transmission; secondly, it provided a source of supply of the complement-fixing and skin-test antigens; and lastly, it seemed to be a possible method of determining the neutralization index of a serum, analogous to that employed in the pock-counting method with herpes and vaccinia viruses. Some evidence was obtained that the present sera reduced the number of lesions on the chorio-allantoic membrane compared with several negative control sera, but again we encountered various technical difficulties. These facts are mentioned here as they seem to warrant further investigation.

Complement-fixing Antibody.—Warren and Sabin (1942) showed that specific complement-fixing antibodies developed in the rhesus monkey during convalescence, but that they did not persist longer than a few months. Obviously if similar antibodies developed in the human, the complement-fixing test would provide a method of diagnosis which would have the advantage of being rapid and would obviate the necessity for animals. The complement-fixing antibody appeared to be more stable than the neutralizing antibody in that it was not inactivated at 56° C. for 30 minutes and the titre persisted for a long time in the refrigerator. Warren and Sabin (1942) obtained fair correlation between positive human neutralizing sera and positive complement-fixation reactions, but the results were not entirely satisfactory. Of 43 sera with neutralizing antibody as many as 20 had no demonstrable complement-fixing antibody. This failure to find complement-fixing antibody may

TABLE I
EXTENT OF RABBIT SKIN LESIONS

Serum	Dilution of Infected Mouse Brain				Result
	1/20	1/100	1/1,000	1/10,000	
V.D. . .	++++*	+++	+	--	Doubtful
Negative serum	++++	++++	+++	+	Negative
Tyrode control	++++	++++	+	--	Negative
Mrs. D.	++++	++	--	--	Positive

* +++++ signifies erythematous indurated lesion with central necrosis on eighth day, an arbitrary end-point at 1/20 in the control series; +++, ++, +, -- represent varying degrees of reaction compared with this control.

have been due to several factors. In the first place the antigen, in this case prepared from rabbit brain, may have been too weak. Secondly, the physical conditions under which fixation was allowed to occur may not have been entirely satisfactory. Thirdly, the titre of the complement-fixing antibody might have fallen (none of the cases was acute) to a level at which it was no longer detectable. Warren and Russ (1948) have recently described a method of cultivation of *Toxoplasma* in the developing chick embryo and the production of a specific complement-fixing antigen from the chorio-allantoic membrane. Using this antigen they obtained results more comparable with those obtained with the neutralizing antibody test, and significant titres were found even in cases which had presumably been infected several years previously.

Complement-fixing Test.—Dr. J. Warren, of the Virus Research Department, Army Medical Department, U.S. Army, Washington, D.C., very kindly sent a sample of complement-fixing antigen, together with some hyperimmune guinea-pig serum as a positive control.

The method employed was that of Warren and Russ (1948). Sera were inactivated at 60° C. for 20 minutes. Serial twofold dilutions of test sera, from 1/2 to 1/256, were made in normal saline. Four units of antigen, previously titrated in the presence of two units of antibody, and two units of complement were added to each tube. Fixation was allowed to take place in the ice box (4° C.) overnight. The haemolytic system, made up of an equal volume of 3% sheep cells and two units of haemolysin in saline, was then added to each tube. Results were read after incubation at 37° C. for one hour; 100% fixation was taken as the end-point. The necessary controls, including a known positive serum, were set up with each test. Normal antigen was prepared from uninoculated chorio-allantoic membrane. The results obtained are shown in Table II.

TABLE II

Serum	Serum Dilution giving 100% Fixation		Result
	Toxoplasma Antigen	Normal Antigen	
V.D.	1/16	0	Positive
Mrs. D. . . .	1/32	0	Positive
Positive control	1/128	0	Positive
Negative control	0	0	Negative

0 = No fixation at serum dilution of 1/2.

One of the many difficulties in any complement-fixation test is the interpretation of the results. In those cases where it is possible to test an acute and convalescent sample of serum and thereby demonstrate a rise in antibody as the result of infection, more significance can be attached to such an increase in antibody than to the antibody level of a single sample. In chronic or healed toxoplasmosis one is invariably testing single samples, taken in some cases many years after infection. In Warren's opinion (1948) a titre of 1/8 is doubtful and 1/16 positive. Furthermore, such titres have greater significance if there is clinical evidence to support the diagnosis. We have been able to examine five sera, three from cases with clinical evidence of toxoplasmosis and two of the mothers. In all five the complement-fixation titre was 1/16 or greater when first tested. The titre did not vary after repeated tests during the ensuing two months. We also examined 200 routine Wassermann sera in order to obtain some idea of the normal antibody level of the population of this country. Of the 200 tested, 175 produced no fixation at 1/2; 10 were anticomplementary, and 12 showed some degree of fixation (in three cases up to 1/12) with both normal and *Toxoplasma* antigens. Three sera, however, gave titres of 1/4, 1/8, and 1/16 respectively, all controls being negative. These cases are being further investigated.

On the other hand, a negative complement-fixation titre (1/2 to 1/8) does not necessarily rule out the possibility of *Toxoplasma* infection. These low titres may be the result of past infection or may be entirely non-specific. Ruchman (1948) has shown that approximately 10% of normal individuals in the U.S.A. have demonstrable neutralizing antibodies in their serum. Frenkel (1948), in a survey of the population of the U.S.A., has shown that 20% of apparently normal people give a positive skin reaction with toxoplasmin. Figures for the incidence of complement-fixing antibodies are not yet available. Until a more detailed survey of normal people in this country has been made it will not be possible to assess the precise significance of a complement-fixation titre of less than 1/8, and for this reason it is advisable to test sera by all available methods before ruling out the diagnosis of toxoplasmosis.

Cytoplasm-modifying Antibody.—The demonstration of this antibody, as described by Sabin and Feldman (1948), depends on the ability of immune serum to modify the staining properties of living toxoplasmas. If a suspension of toxoplasmas from mouse peritoneal exudate is subjected to the action of methylene blue the parasites

take up the stain and at the same time their crescentic shape is altered towards the spheroidal. If, however, toxoplasmas are first incubated with immune serum and then methylene blue is added, a proportion of them, depending upon the strength of the antibody, retain crescentic shape and fail to take up the stain. In a positive case all the toxoplasmas are unstained in the higher concentrations of serum, except that a prozone may be seen in the greatest strengths, while all take up the stain when the antibody is sufficiently diluted out. By suitable dilution a titre of the serum may be ascertained at which 50% of the toxoplasmas are stained, which is regarded as the end-point of the reaction. Fig. 1 shows both stained and

FIG. 1.—Results seen in a cytoplasm-modifying test. The arrows indicate unstained toxoplasmas. $\times 300$.

unstained toxoplasmas. The unstained ones are so feebly refractile that it is almost impossible to photograph them, and the photographs of Sabin and Feldman of unstained toxoplasmas do not, in our experience, represent the completely unstained crescents which we consistently see with immune sera.

Sabin and Feldman (1948) have shown that although the antibody is apparently very labile, in fact it is reasonably stable, and when potency has been lost it may be restored by the addition of normal serum. They describe an "accessory factor," different from complement, which is sparingly present in human serum and very labile, whose presence is necessary for the cytoplasm-modifying antibody to act.

For the performance of the test the toxoplasmas must be used within an hour of being removed from the mouse. The antibody works slowly at room temperature, but its action is complete after one hour at 37°C . Methylene blue must not be older than three to four days, and for immediate staining Sabin and Feldman recommend a highly alkaline preparation, either 3 ml. of saturated alcoholic solution of methylene blue with 10 ml. of alkaline soda-borax buffer solution of pH 11 (9.73 ml. of 0.53% Na_2CO_3 plus 0.27 ml. of 1.9% $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) or a freshly prepared 0.25% methylene blue solution in the pH 11 buffer.

Four-day-old peritoneal exudates are used, because at this time they contain the greatest number of extracellular toxoplasmas and because they have not yet been affected by the immune response of the host. Immediately after removal from the mouse peritoneum the exudate is diluted 1:5 with heparinized saline or serum to prevent the deposition of fibrin. Serial dilutions of the serum to be tested are made using a normal human serum to provide "accessory factor," and to these are added equal volumes of *Toxoplasma* suspension. After incubation for one hour at 37°C . a drop from each dilution is mixed with half its amount of methylene blue and examined under a cover slip at a magnification of 475 for counting the numbers of stained and unstained parasites.

Adhering rigidly to these instructions of Sabin and Feldman, we were unable to make the test work at all, presumably for a variety of reasons, some of which were clarified. First, with the *Toxoplasma* suspension diluted 1:5 with saline there was apparently an insufficiency of "accessory factor," and this was corrected by making the dilution in normal human serum. Secondly, for some unexplained reason we found that methylene blue solutions used on the day of preparation, which was our original practice, stained the toxoplasmas so faintly that a reliable differential count of stained and unstained parasites was unobtainable. Next, it was found that the recommended alcoholic methylene blue solution produced a deposit in the *Toxoplasma* suspension which rendered the parasites uncountable. Lastly, as luck would have it, the first donor serum used for diluting purposes yielded preparations in which none of the toxoplasmas was stained, although repeated several times, and eventually we found that this donor had a cytoplasm-modifying antibody in his serum to a titre of 1/16.

Cytoplasm-modifying Test.—The technique finally adopted, which differs in detail but in no way fundamentally from that of Sabin and Feldman, is as follows.

Four-day mouse peritoneal exudate is taken immediately into an equal volume of normal human serum containing 100 units of heparin per ml. (with greater dilutions of the exudate we are unable to obtain sufficient extracellular toxoplasmas to permit of easy counting). To 1 volume of serial dilutions of the suspect serum in normal serum is added 1 volume of the exudate, and the mixtures are incubated in a water-bath at 37° C. for one hour. After removal from the bath, 1 volume of 0.25% methylene blue in pH 11 buffer is added to each tube and counts are made after standing for a further 10 minutes on the bench. The methylene blue must not be less than two days old, and we have found that it stains well up to 14 days.

Occasionally for no apparent reason the whole test is a failure, all the parasites staining, none of them staining, or all staining so faintly that no differentiation can be made. For this reason we always include with the titrations controls of *Toxoplasma* suspension to ensure that the methylene blue is working, and a known positive serum titrated out beyond its end-point.

When counting the preparations the unstained toxoplasmas are often very difficult to see, which probably leads to an error in favour of the stained ones. To avoid any prejudice the counter does not know which serum or dilution he is examining. Intracellular parasites stain whether immune bodies are present or not, and are excluded from the differential count.

The simplicity of this test, when all goes smoothly, is only offset by the necessity for maintaining a strain of living *Toxoplasma* in the laboratory. In acute disease and in recent cases Sabin and Feldman found titres up to 1/16,348,

FIG. 2.—Curves obtained by cytoplasm-modifying test (Mrs. D.) made on three different occasions.

FIG. 3.—Curves obtained by cytoplasm-modifying test (V.D.) made on three different occasions.

whereas when the history suggested an infection six to seven years previously titres of 1/16 to 1/64 were present.

Both Mrs. D. and V.D. showed the presence of cytoplasm-modifying antibody, the former to a titre of 1/128, the latter to a titre between 1/64 and 1/128. Figs. 2 and 3 show the curves obtained in each case when the test was repeated on three different occasions. They both show a prozone, the greatest number of unstained toxoplasmas not being seen until a dilution of 1/16. All the eight positive sera we have been able to test have shown this zoning phenomenon, and for this reason a full range of dilutions must always be set up, as a single tube test for presumptive diagnosis might fall in the prozone. It will be observed that although the total number of toxoplasmas remaining unstained varies from test to test the dilution at which 50% of them are unstained (the end-point of the titration) is remarkably constant.

Thirty control sera have not shown the presence of cytoplasm-modifying antibody, with the one exception of the donor whose serum was used as a diluent in our early efforts to make the test work and showed an antibody titre of 1/16 when diluted out with other normal serum. Whether this is a non-specific reaction or denotes a previous infection is not clear, as the donor shows no clinical evidence of past infection and his serum is persistently anticomplementary.

Diagnostic Use of Toxoplasmin

Warren and Russ (1948) and Frenkel (1948) have recently described the use of toxoplasmin, a skin-test antigen, as an aid to the diagnosis of toxoplasmosis. Toxoplasmin can be prepared either from the chorio-allantoic membrane of the chick embryo or from *Toxoplasma*-infected mouse peritoneal fluid. In each case control antigens are prepared from normal uninfected tissues. After suitable preparation such antigens, which are in the nature of soluble antigens and are in fact the same as the complement-fixing antigens, have the property of producing a specific reaction when injected intracutaneously into sensitized people; the reaction appears to be similar to the Mantoux reaction. Frenkel (1948) has shown that most patients with a positive neutralizing serum give a positive skin test with toxoplasmin, and that many cases in which the neutralization test has given a doubtful result also give a positive skin reaction. Toxoplasmin also appears to act as a "booster" to weak sera, as several were found to be positive at a later date. This did not occur with normal individuals inoculated with toxoplasmin.

Toxoplasma skin-test and control antigens were prepared according to the methods described by Frenkel (1948) and Warren and Russ (1948). The mouse peritoneal fluid antigen diluted 1:10,000 was used on V.D. and on three apparently normal children. An area of erythema 1 x 1 cm. developed on V.D.'s arm after 24 hours, but did not persist longer than 48 hours. The control children were completely negative. This was regarded as a doubtful result, and as such clearly needs further investigation.

The difficulties inherent in all the laboratory tests for toxoplasmosis are notorious. Earlier attempts to establish the rabbit neutralization test in this laboratory ended in failure mainly owing to the difficulty in interpreting the results. Similarly, the initial trials of the cytoplasm-

modifying test were most disappointing, and only the clear-cut findings with the complement-fixation test led us to persevere until the snags had been eliminated.

From the nature of the case presented here attempts to isolate *Toxoplasma* were foredoomed to failure. But with the clear correlation between the neutralizing, complement-fixing, and cytoplasm-modifying antibody tests we feel we are justified on laboratory findings alone in making the diagnosis of a previous *Toxoplasma* infection. In the presence of a clinical history suggestive of the disease these findings assume added significance.

Summary

The laboratory methods available for the diagnosis of toxoplasmosis are the isolation of the *Toxoplasma*, the demonstration of neutralizing, complement-fixing, and cytoplasm-modifying antibodies, and the production of a skin reaction with toxoplasmin.

These investigations are described and discussed, and ways of avoiding some of the technical difficulties are indicated.

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