STAINING TOXOPLASMA GONDII WITH FLUORESCEIN-LABELLED ANTIBODY

I. The Reaction in Smears of Peritoneal Exudate

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Toxoplasma gondii is a small organism which ordinarily is difficult to identify in tissue sections unless it occurs in clusters known as pseudocysts. A staining method specific for the organism would therefore be of value in the microscopic study of lesions suspected of being caused by this species. Since fluorescein-labelled antibody can be used to stain homologous organisms specifically (reference 1, contains extensive bibliography) a study was undertaken to see whether *Toxoplasma* could be stained by this method. Preliminary experiments showed that *Toxoplasma* in smears prepared from peritoneal fluid or organs of infected mice were readily stained with labelled antibody. Some details of this staining reaction were then studied in order to develop methods which would facilitate serologic and other investigations on toxoplasmosis. The first paper in this series describes the general methods used and the information obtained about the antigen complex which is demonstrable with labelled antibody. In the second paper, which follows immediately, the use of this staining method as the basis for a serologic test for *Toxoplasma* antibodies is described. Subsequent reports will deal with the problem of staining the organisms in tissue sections.

Materials and Methods

Antibody.—A human serum with a titer of 1:4000 in the methylene blue dye test (2) was fractionated with half-saturated ammonium sulfate in order to obtain the globulin portion. There was no significant difference in the dye test titer between the original serum and its globulin portion. The globulin was labelled with fluorescein (3) and the conjugated protein was dialyzed and repeatedly precipitated with half-saturated ammonium sulfate to remove uncombined fluorescein. The final product was dialyzed against slightly alkaline buffered saline and kept at −20°C. when not in use.

Other workers have found that such conjugates needed to be absorbed with tissue powders before use in order to remove so called "non-specific staining." In the work to be described here it was found that for ordinary staining of smears of peritoneal exudate such absorption was not necessary, possibly because of the relative absence of tissue elements that might have taken up the stain. However, when smears were stained in the serologic test reported in the second paper of this series, it was found that conjugates prepared from at

least one serum sample gave better results after being absorbed with liver powder. The method of preparing the liver powder used in this work differed in detail from that described by Coons, Leduc, and Connolly (4) but the principle was similar and no attempt was made to compare the relative effectiveness of the two products.

Antigen.—Toxoplasma gondii organisms, strain RH, were obtained from mice infected 3 or 4 days previously by the peritoneal route. Early experiments included staining dab smears of organs as well as smears of peritoneal exudate. Since results appeared to be the same in both cases, and since organisms were more conveniently obtained from exudate, subsequent work was done entirely with peritoneal fluid. The method finally adopted for preparing large numbers of smears for staining experiments was as follows: 0.5 to 1.0 ml. of peritoneal exudate was discharged into 10 ml. of 1.0 per cent formalin in 0.85 per cent saline. After 30 minutes at room temperature, the suspension was spun at about 500 r.p.m. for 5 minutes to throw down heavier particles present in the exudate. The supernate was then poured into another tube and spun at 1,500 r.p.m. for 5 minutes to throw down the toxoplasms. The supernate was discarded and the sediment was resuspended in about 3 ml. of saline. Smears were made by allowing small drops of this suspension to dry on slides over an area about 4 to 5 mm. in diameter. When the smears were dry, the slides were stored in a freezer held at approximately −20°C. For use, slides were removed from the freezer and allowed to dry thoroughly before adding the staining solution.

Staining Procedure.—0.04 ml. of diluted or undiluted labelled antibody was placed onto a dried smear which was then put into a wet chamber at 37°C. After 1 hour, the slide was removed and washed in saline and tap water for 10 and 5 minutes, respectively. After being allowed to dry, a drop of alkaline buffered glycerin (9 parts of glycerin plus 1 part of 0.15 M phosphate buffer, pH 8.0) was added, and a coverslip was applied.

Fluorescence Microscopy.—For most of this work, a General Electric AH-6 mercury vapor lamp was the light source.[1] It was mounted horizontally in a modified Bausch and Lomb research lamp housing which was set directly beneath the microscope, eliminating the need for a mirror. The microscope was equipped with a cardioid darkfield condenser and apochromatic objectives. A Corning glass filter, No. 5850 (4 mm.), was used to give an input light of predominantly blue, red, and near ultraviolet wave lengths. Corning yellow and green filters, Nos. 3486 (2 mm.) and 9780 (5 mm.) respectively, were both used in the eyepieces to isolate the characteristic yellow-green fluorescence of fluorescein. With this system, it was possible to observe results with a binocular head on the microscope. For maximum brightness a monocular head was used, together with a Klett 42 filter or 2 thicknesses (8 mm.) of Corning No. 5850 to control the input light. It is worth noting that fluorescein absorbs blue light to a greater extent than near ultraviolet light (5) and, therefore, it fluoresces more brightly when the exciting wave lengths are in the blue range.

For part of this work, a Leitz fluorescence lamp assembly[2] was used in conjunction with a standard, monocular microscope equipped with an American Optical Company darkfield condenser. This combination was very satisfactory although results were not as bright as with the other set-up.

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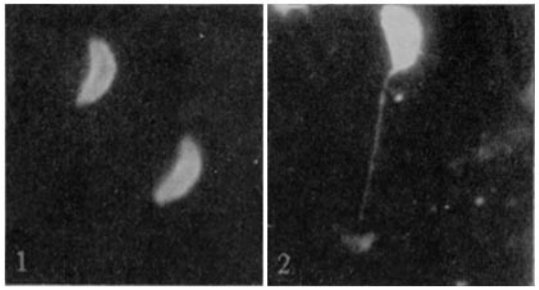
[1] Trade names are used for purposes of identification only and do not constitute endorsement by the United States Public Health Service.

[2] Thanks are due to Mr. W. F. Butler of the Leitz Compay for kindly arranging the loan of this equipment.

EXPERIMENTAL AND RESULTS

Although the shape and size of stained toxoplasms varied somewhat with the method of fixation, the fluorescent staining picture observed following exposure to labelled antibody was more or less the same in all cases (Fig. 1). Fluorescence tended to be brighter on the periphery of each organism than in the center and frequently the polar regions were brighter than the central portion.



Both photographs were taken on the fluorescence microscope using a 4 mm. fluorite, oil immersion objective and a 20 × eyepiece, with a nominal magnification of 1000. Exposure time was 2 minutes using 35 mm. tri-X film developed in microdol.

FIG. 1. Characteristic appearance of toxoplasms from mouse peritoneal exudate after staining with fluorescent antibody.

FIG. 2. Stained toxoplasm from exudate washed with 0.1 per cent formol-saline, showing thread-like structure emanating from pointed end of the organism.

1. Evidence of Specificity

The specificity of the staining which was observed was established by the following controls:

(*a*) Toxoplasms exposed to labelled human and labelled rabbit sera, both negative for *Toxoplasma* antibodies as determined by the dye test, did not become fluorescent; however, when exposed to labelled antiserum under the same conditions they became brightly fluorescent.

(*b*) Staining was inhibited by exposing organisms to non-labelled antiserum first, and then to labelled antiserum. Inhibition did not occur when organisms were exposed first to non-labelled normal serum and then to labeled antiserum.

One experiment was conducted to see if labelled antitoxoplasma serum would stain a possibly related organism. Three strains of *Trypanosoma cruzi* flagellates obtained from culture were air-dried on slides. Three days later they were fixed with 10 per cent formol-saline for 15 to 30 seconds, and then exposed to labelled antitoxoplasma serum and labelled normal serum. In both cases a low level fluorescence was observed distinctly different from the appearance of specifically stained *Toxoplasma* organisms.

2. *Staining Activity of Toxoplasms after Various Treatments*

(*a*) *Effect of Different Fixatives.*—

Smears of peritoneal exudate were allowed to air-dry on slides and some were then immersed in one of each of the following solutions for 15 minutes: 10 per cent formalin, absolute methanol, and acetone; other smears were left untreated. Following this, they were rinsed briefly and exposed to labelled antiserum. In other experiments, fresh exudate was discharged into separate tubes containing 0.85 per cent saline, 0.1 per cent formalin in saline, 1.0 per cent formalin in saline, and 0.1 per cent phenol in saline. The saline suspension was heated at 56°C. for 30 minutes; the other suspensions were left at room temperature for from 10 to 30 minutes. After this all suspensions were centrifuged, the sediments were resuspended in saline and smears were prepared from them. The smears were then exposed to labelled antiserum.

In all cases, organisms became brightly fluorescent following exposure to labelled antibody. Although it was difficult to quantitate the degree of fluorescence in these smears, it appeared that heat-treated organisms were not as bright as organisms not subjected to heat. Smears fixed with 10 per cent formalin in water (instead of saline) showed organisms with fluorescent, granular deposits on what appeared to be the outside of the cell membrane. When the formalin solution was made up with saline, the organisms appeared similar to those fixed by other methods. Organisms in fresh exudate washed with 0.1 per cent formol-saline and then stained, showed typically one or more fluorescent, thread-like structures emanating from the pointed end of the organism (Fig. 2). The threads were less common in material fixed with 1.0 per cent formol-saline, and were entirely absent when 10 per cent formol-saline was used. The nature of these threads has not been determined, but the results of an investigation of the subject will be reported elsewhere.

In the experiments described above, organisms were exposed to fixatives for less than 1 hour. In order to determine the effect of longer formalin fixation on the staining reaction, infected exudate was first exposed to ten times its volume of 1.0 per cent formol-saline for 30 minutes, washed with saline, and then portions were resuspended in saline and in 1.0 per cent formol-saline for 2 hours. At half-hour intervals during the 2 hour period portions were removed and dried smears were prepared from them. When these smears were exposed to labelled antibody, there was a distinct and progressive decrease in the staining of organisms exposed to formalin, whereas organisms exposed to saline showed no change in their staining reaction.

(*b*) *Effect of Time and Temperature.*—The initial staining experiment in this work was done with smears prepared 21 days previously and kept at room temperature during the entire period. Organisms stained very well regardless of whether they had been chemically fixed or not immediately after the smears had dried. This result turned out to be a fortunate accident because, later, it was found that smears lost their ability to be stained after

varying periods of time, depending especially upon the temperature at which they were stored, although other details of handling the organisms also affected their staining ability.

Smears prepared directly from unwashed peritoneal exudate and kept at room temperature usually remained reactive for about 3 weeks. Beyond that time, their reactivity declined so that by the 36th day no staining resulted following exposure to labelled antiserum. Occasionally, organisms stained poorly within 1 week after smears were prepared, but this was unusual. The pattern of change in smears kept at room temperature was one in which organisms became progressively less reactive with labelled antiserum, while the background and white blood cells showed progressively more primary fluorescence even without exposure to labelled serum.

In order to compare the reaction of organisms kept at room temperature with those kept at a lower temperature, smears were prepared from unwashed exudate and some were put into a −20°C. freezing cabinet; others were left at room temperature. After 23 days both types of smears were fixed with methanol and exposed to labelled antiserum. Those kept at room temperature were very poorly stained; those kept at −20° stained as well as freshly prepared smears. Although repeated tests showed that keeping smears at −20°C. extended their reactive period well beyond that of smears kept at room temperature, some variation in activity occurred here too. For example, a batch of slides prepared from one exudate lost its activity by the 27th day in the freezer whereas other batches prepared from other exudates were still good at 36 days.

Previous work with toxoplasms had indicated that the liquid portion of the peritoneal exudate obtained from infected mice was a factor of importance in the behavior of toxoplasms in serologic reactions (6, 7). In addition, the obvious difference in viscosity of fluids obtained from different mice affected the character of smears prepared from these exudates. Therefore, attempts were made to improve the reproducibility of staining experiments by washing the organisms free of exudate. Infected peritoneal exudate was drawn and smears were made directly with some of it. The rest of the exudate was discharged into 10 volumes of 0.1 per cent formol-saline, mixed thoroughly and then centrifuged. After pouring off the supernate and adding a little saline, smears were prepared from the washed, sedimented organisms. Slides from both sets of smears were fixed and stained on the day of preparation and after 6 days at room temperature. Smears of unwashed exudate showed considerable background fluorescence, and the staining of organisms was much reduced in the 6 day old preparations. Smears of washed exudate showed clear dark backgrounds with bright staining organisms in both fresh and 6 day old preparations. Subsequent experiments showed that smears prepared from exudates washed with 0.1 or 1.0 per cent formol-saline, and kept

at −20°C. retained their staining ability for several months at least. It was also found that such smears, when removed from the freezer and kept at 5°C. retained their staining ability for at least 57 days, but when they were transferred to room temperature they stained poorly after about 28 days. When smears of washed exudate were kept at 5°C. all the time, the organisms stained well for as long as 28 days but their morphology was less precise and the background staining was greater than comparable smears kept at −20°C.

3. Viability of Formalin-Washed Toxoplasms

One of the reasons for using formalin-washed organisms in this work was to reduce the hazard involved in handling exudates containing infectious *Toxoplasma*. Several experiments were conducted to see whether the washing treatment actually did render the organisms non-infectious.

A preliminary experiment showed that exudate containing an unknown number of organisms exposed to 10 volumes of 0.1 per cent formol-saline for 10 minutes was lethal to mice in from 13 to 15 days. The experiment was repeated using four groups of five mice each which received intraperitoneal inocula as follows:

Group A—1,360,000 toxoplasms per mouse; Group B—6,800,000 toxoplasms per mouse; Group C—20,400,000 toxoplasms per mouse; Group D—No toxoplasms, saline only.

Each group, except for the negative control, was set up in duplicate, one set receiving toxoplasms exposed to 0.1 per cent formol-saline for 10 minutes, and the other receiving organisms exposed for 20 minutes. Within 6 days essentially all the mice, except the negative controls, were dead.

In view of the fact that 0.1 per cent formol-saline did not kill all of the toxoplasms under these conditions, another experiment was set up in which one aliquot of *Toxoplasma*-containing exudate was exposed to 1.0 per cent formol-saline for 30 minutes at room temperature. Each of 5 mice (Group A) received 5,400,000 toxoplasms intraperitoneally. In addition, 5 other mice (Group B) received the same number of organisms exposed to 0.1 per cent formal-saline for 30 minutes, and 5 others (Group C) received saline inoculations. Five days later, all five mice in Group B were dead. In Group A, 1 mouse died on the 5th day. Twelve days later, all 4 remaining mice in Group A and all 5 mice in Group C were alive and well.

DISCUSSION

It is believed that the control experiments cited above adequately support the view that the bright fluorescent staining observed in this work was due to an antigen-antibody reaction. The attempt to stain *T. cruzi* with anti-toxoplasma serum was undertaken because of a report (8) that *T. cruzi* infections in mice stimulated the production of antibodies effective

against *T. gondii* in the methylene blue dye test. Although our results were negative, the single experiment was too fragmentary to be conclusive.

The distribution of fluorescent material within stained toxoplasms cannot be taken as necessarily indicating the location of antigen in the living cell. It can be readily demonstrated with an ordinary Giemsa stain that different methods of fixation and drying will yield very different-looking organisms, and it is very probable that diffusion of at least some internal contents takes place when the fixation and staining methods employed in this work are used.

The characteristics of the antigen system that was involved in the staining reaction may be summarized as follows: It was not affected by brief exposure to methanol, acetone, dilute or concentrated formalin, or dilute phenol. It appeared to be somewhat susceptible to higher temperatures, although it was not destroyed by exposure to 56°C. for 30 minutes. Keeping qualities of the antigen were greatly improved by maintenance at −20°C. Uniformity of activity from one batch of organisms to another, and also the keeping qualities appeared to be improved by washing the toxoplasms in dilute formalin.

It has been shown (9) that as few as 200 toxoplasms are capable of producing lethal infections in mice in 9 days. In the work reported here as many as 5,400,000 organisms exposed to 1.0 per cent formol-saline for 30 minutes were not capable of killing 4 out of 5 mice in 12 days. This indicates that the washing method used was adequate to inactivate most if not all of the organisms. The possibility remains that some organisms, particularly those enclosed in leucocytes, might survive brief exposure to dilute formalin, and might be capable of setting up inapparent infections in mice that could be uncovered only by serial passage of organs from inoculated mice. This possibility was not tested since it was felt that even if such a degree of infectiousness was present, it was too low to be of practical importance under the conditions used in this work.

From the results reported here, it would seem that the method of staining organisms with labelled antibody may be a valuable approach in characterizing antigen systems in micro-organisms. Since relatively few cells are needed in any one test, one could, for example, check the susceptibility of an antigen to a battery of enzymes in a fraction of the time that would be required for similar tests to be run on extracts of organisms.

SUMMARY

Antitoxoplasma globulin was labelled with fluorescein and was used to stain *Toxoplasma gondii* organisms in smears of peritoneal exudate. The evidence indicates that this staining was due to an antigen-antibody reaction at the cellular level. Methods are presented for handling the organisms so

that they can be stained over a period of several months. Some general characteristics of the antigen system involved are described.

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