STAINING TOXOPLASMA GONDII WITH FLUORESCEIN-LABELLED ANTIBODY

II. A New Serologic Test for Antibodies to Toxoplasma Based upon Inhibition of Specific Staining

By MORRIS GOLDMAN, Sc.D.

(From the United States Department of Health, Education, and Welfare, Communicable Disease Center, Atlanta)

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The serologic test commonly used at present to detect antibodies to *Toxoplasma* is the methylene blue dye test (1). This method depends upon the ability of specific antiserum to modify live toxoplasms so that they no longer stain with a methylene blue solution. Two important difficulties associated with this test are that it employs a live antigen obtained from infected mice just before use in the test, and that it requires the presence of a complement-like substance, "accessory factor," which is obtained from human serum. In addition, the experience of several laboratories (2–5) has been that the test is capricious and cannot always be depended upon to yield valid results as a routine, diagnostic method. For this reason, the potential usefulness of a test which would yield results parallel to those of the dye test, and which would avoid the difficulties associated with the latter method would be great. The fluorescence inhibition technique, described in this paper, seems to offer possibilities as a routine screening procedure to be followed up by the dye test on detected positives only. From the standpoint of the clinical diagnostic laboratory, such an approach would greatly simplify the testing of sera for evidence of toxoplasmosis. It was from this standpoint that the work described here was performed.

Coons and Kaplan (6) demonstrated that it is possible to block the staining of antigen with homologous labelled antibody by first exposing the antigen to the original non-labelled antibody. This test for specificity of staining has been widely used in fluorescent antibody studies. When the test was applied to the staining of *Toxoplasma gondii* with labelled antibody (7), inhibition of fluorescence was very marked, and the possibility was then considered that antibody might be detectable in unknown sera by observing the degree of inhibition which such sera demonstrated. The purpose of this paper is to describe the serologic test for toxoplasmosis which was developed on the basis of this inhibition phenomenon, and to present evidence concerning the specificity and reliability of the method.

Materials and Methods

Labelled Antibody.—Two labelled sera were used in this work: T-1, a human serum showing a 1:4000 titer for toxoplasmosis in the methylene blue dye test, and T-3, a pool of sera taken from experimentally infected rabbits.[1] The globulin fraction of this pool had a dye test titer of 1:16,000.

The globulin portions of these sera were conjugated with fluorescein by the method of Coons and Kaplan (6). When not in use, conjugates were kept frozen at −20°C.

Antigen.—Smears of *T. gondii* were prepared from peritoneal exudate of 3- or 4-day infected mice. The exudate was washed with 0.1 or 1.0 per cent formol-saline, using methods described previously (7). Depending upon the number of smears prepared and the number of tests run, smears made from the same batch of exudate were used for up to 1 month. When not in use, smears were kept at −20°C.

Test Sera.—These were primarily human sera submitted for diagnosis to the Toxoplasma Diagnostic Laboratory of the Communicable Disease Center over a period of about 1 year. The sera were kept frozen at −20°C. until used. In most cases, dye tests were also run on these specimens.[2] In addition, a group of sera from laboratory and other animals was tested. Sera varied in character from fresh, sterile, non-hemolyzed samples to year old, badly contaminated, and badly hemolyzed specimens, but no controlled studies were carried out on the influence of these factors upon the test. Sera that contained much suspended material were centrifuged at high speed before being tested. Except for a few preliminary experiments in titration, all tests were done with undiluted sera.

Performance of Inhibition Tests.—In some earlier experiments, variations of the following basic technique were used; however, only the method adopted finally is given here. Smears were removed from the freezer and allowed to dry thoroughly. Test serum in the amount of 0.02 ml. was mixed in a tube with 0.02 ml. of labelled antibody, usually diluted 1 + 2 or 1 + 4, and the entire mixture was transferred to a smear by means of a bulb-operated pipette. The dilution of labelled antibody varied with different batches of smears and was determined for each batch by a titration procedure described in the next section. At first the diluent used was 0.85 per cent saline, but later 2.5 per cent bovine serum albumin in 0.85 per cent saline was substituted when it was found that results tended to be more clean cut with the latter solution. Smears were put into a wet chamber at 37°C. for 1 hour, removed, and immersed in saline and tap water for 10 and 5 minutes respectively. They were then dried, mounted with buffered glycerol and examined with the fluorescence microscope described previously (7).

The degree of fluorescence was recorded by plus marks according to the following guide:—

±—no definite fluorescence, organisms only vaguely visible, indicating strong inhibition;

1+—definite fluorescence, color non-descript, not definitely green;

2+—definite fluorescence, color definitely yellow-green but of low intensity;

3+—bright yellow-green color, sharp, and clear;

4+—brilliant yellow-green color, very sharp, indicating no inhibition.

In order to help the observer determine the amount of fluorescence with some degree of objectivity, reflector type neutral density filters were placed in the path of the input light. With the fluorescence system used in this work, it was found that a 0.6 density filter greatly reduced the yellow-green color of a 2+ fluorescence, but allowed the color of 3+ brightness to come through rather clearly.

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[1] This serum was made available through the kindness of Dr. Leon Jacobs of the National Institutes of Health, Laboratory of Tropical Diseases.

[2] Dye tests were performed by Mr. A. J. Sulzer and Miss Ruth Gogel to whom the author is greatly indebted for access to sera and dye test results.

All sera were tested twice as unknowns, and in the earlier experiments they were read independently by two individuals. Negative and positive control sera were set up in every run, fluorescent results usually being 3–4+ and 1–2+, respectively.

EXPERIMENTAL

One-Step Inhibition Test.—Inhibition tests used in the past to prove the specificity of fluorescent staining with labelled antibody have been of the sequence type; *i.e.*, antigen has been exposed first to unlabelled antibody, rinsed, exposed to labelled antibody, rinsed again, and examined (6). The first tests performed in the present work with toxoplasms were of this type, smears being covered with unlabelled antiserum for 1 or 2 hours and then

TABLE I

Fluorescent Staining Obtained with the Sequence and Combined Method for Demonstrating Inhibition

Type of smear	Sequence method		Combined method	
	Positive serum followed by labelled antiserum (T-1)	Negative serum followed by labelled antiserum (T-1)	Labelled antiserum (T-1) plus positive serum	Labelled antiserum (T-1) plus negative serum
Original exudate	1–2+	2+	±–1+	3–4+
Exudate washed with saline	1–2+	2+	±–1+	3+
Exudate washed with 0.1 per cent formol-saline	2+	3+	1+	4+

with labelled antiserum for 1 hour. Generally, but not always, inhibition was demonstrated by this method. Reasons for partial or complete failures of the test were obscure, but positive results were frequent and clear enough so that in a preliminary group of 49 sera tested as unknowns, agreement with the dye test occurred in about 85 per cent of the cases. At this point a new batch of smears was prepared and 24 more sera were tested. In this group, agreement between dye and inhibition test results dropped to 69 per cent with some serious discrepancies occurring between the results of the two tests. Another batch of exudate was obtained and smears were prepared from the original exudate, from exudate washed with saline, and from exudate washed with 0.1 per cent formol-saline. An inhibition test performed on these smears, using the sequence method of exposure, showed very little difference between the effects of known positive and negative sera. However, when the inhibition test was performed by a one-step procedure in which the unlabelled and labelled sera were combined and applied to the smears together (8), it was obvious that the one-step, or combined method demonstrated inhibition much more clearly than the sequence method (Table I). Six new sets of smears,

prepared from different batches of exudate on different days and stained by the combined method, showed clear inhibition in all cases, indicating that the method was reasonably dependable. Since, in addition to being more dependable, the combined method was also simpler and quicker to perform than the older test, it was decided that all subsequent testing of sera would be by this method.

Exploratory experiments with the combined method showed that the inhibition effect was a quantitative matter; *i.e.*, by varying the relative concentrations of labelled and unlabelled antisera one could obtain greater or lesser inhibition. It was therefore necessary to titrate the labelled antiserum so that the greatest contrast would be developed when the serum was mixed with known positive and negative sera. A typical protocol is shown in Table II. In this case, two sera positive in the dye test at different titers, and one

TABLE II

Fluorescent Staining Obtained in Titrating Labelled Antiserum in Order to Demonstrate Optimum Degree of Inhibition

Sera added to labelled antiserum (dye test titers shown in parenthesis)	Original Dilution of labelled antiserum (T-1)			
	Undiluted	1 + 1	1 + 2	1 + 3
369 (1:64)	4+	2-3+	1-2+	1+
475 (1:16)	2+	1+	±-1+	±
Olden (negative undiluted)	4+	3-4+	3+	2-3+

serum negative undiluted in the dye test, were set up in combination with varying dilutions of the labelled antiserum. It can be seen that when the labelled antiserum was undiluted, little or no inhibition was demonstrated by the positive sera, but when the labelled antiserum was diluted out, inhibition became clearly visible. A practical limit to this dilution was set by the intensity of fluorescence in the presence of negative serum, so that in the 1 + 3 dilution, for example, the presumably non-inhibited fluorescent staining was too low for adequate observation and comparison with inhibited reaction. From this titration, a 1 + 2 dilution of labelled antiserum was taken as a proper concentration for testing. It should be noticed that No. 475 was the weaker of the positive sera according to the dye test, but was the stronger inhibitor in the fluorescence test. Subsequent tests confirmed the finding that the dye test titer of a serum was not always directly correlated with the degree of inhibition shown by the same serum. Whenever new smears were prepared, a titration was run using previously determined positive and negative sera as controls. Although smears from any one batch of exudate remained constant in activity at least over the length of time they were usually used for testing, different batches of exudate frequently required slightly different dilutions of labelled antiserum in order to demonstrate inhibition.

In smears showing no inhibition at all and in smears showing strong inhibition, essentially all the toxoplasms were brightly fluorescent or non-fluorescent, respectively. However, in partially inhibited smears, one could frequently find on the same smear organisms that varied in brightness within 1 + mark. Generally the distribution of fluorescent material in an organism was rather uniform except for somewhat brighter staining peripherally.

TABLE III

Relationship of Dye and Fluorescence Inhibition Tests in Two Groups of Randomly Selected Sera

Test used	Fluorescence inhibition test results	No. of sera at indicated dye test titer				No. of sera tested
		<16	1:16	1:64	1:256 and higher	
		GROUP A				
Dye test		44	21	20	15	100
Fluorescence inhibition test 1	Negative	28	6	1	1	36
	Doubtful	4	4	3	1	12
	Positive	12	11	16	13	52
Fluorescence inhibition test 2	Negative	37	9	6	0	52
	Doubtful	5	3	3	6	17
	Positive	2	9	11	9	31
		GROUP B				
Dye test		18 (36)*	12 (24)	12 (24)	8 (16)	50
Fluorescence inhibition test 1	Negative	13 (26)	5 (10)	5 (10)	0	23
	Doubtful	2 (4)	0	3 (6)	1 (2)	6
	Positive	3 (6)	7 (14)	4 (8)	7 (14)	21
Fluorescence inhibition test 2	Negative	11 (22)	4 (8)	3 (6)	0	18
	Doubtful	3 (6)	0	4 (8)	0	7
	Positive	4 (8)	8 (16)	5 (10)	8 (16)	25

* Figure in parenthesis is the percentage based on 50 sera tested.

Comparison of Inhibition, Dye, and Complement-Fixation Tests.—One hundred sera chosen at random, (group A), were tested twice in groups of 20 to 30 at a time. Exposure time in this group was 30 minutes at 37°C. The distribution of the two sets of fluorescence inhibition results as related to dye test titers is shown in Table III.[3] It was obvious that correlation between the two types of tests and between duplicate inhibition tests was poor. A critical

[3] In accordance with recommended procedures for performing the dye test for diagnostic purposes, the lowest dilution used in the test was 1:16. Sera negative at this level have been designated <1:16 in the tables and text. This group, then, includes sera which might have shown positive titers in the lower dilutions or might have been negative undiluted.

check was then made of technical aspects of the inhibition test and several changes were instituted. Care was taken to make sure that smears removed from the freezer for use were very thoroughly dried before exposure to the test mixture; smears were made smaller than before to assure adequate exposure of all organisms; and exposure time at 37°C. was increased to 1 hour so that the amount of time elapsing between exposure of the first and last smears of a set would be a smaller percentage of the incubation period.

Using the modified procedure, a new group of 50 random sera, (group B), was tested in duplicate. In this series agreement between duplicate inhibition tests was closer than before in all categories (Table III). The total number of positives in the first test was 21 (42 per cent), and in the second test 25 (50 per cent), whereas comparable figures for the results of the first 100 sera were 52 and 31 per cent. Thus, it appeared that the changed methods of setting up the tests had considerably improved reproducibility of results. However, correlation between dye and inhibition test results was still poor in the group of sera with dye test titers less than 1:256.

Inasmuch as the 150 sera in groups A and B were chosen at random, there did not always exist sufficient clinical information to evaluate the results of the serological tests. In order to gain more definite information as to the relationship of the inhibition test to actual infection with toxoplasmosis, two new groups of sera were tested (Table IV). In group C, 25 sera were selected in which the clinical history was suggestive of congenital toxoplasmosis and in which the dye test results supported such a diagnosis. Duplicate inhibition tests in this group gave the same results in all but 2 cases which were both positive in one test and doubtful in the other. In addition, agreement with the dye test was much better than in groups A and B, the only difference occurring with serum 217 which had a titer of $<1:16$ in the dye test but was positive in the inhibition test. This discrepancy, and others which occurred in the next group tested, will be taken up in the Discussion.

In group D, 25 sera were selected from patients with histories suggestive of congenital toxoplasmosis, basing this mostly on the presence of eye lesions, but where the dye test results did not always confirm such a diagnosis. Duplicate inhibition tests gave the same results in 20 cases; in the other 5 cases, results were doubtful in one test and either positive or negative in the duplicate test. Out of 5 cases positive at 1:16 in the dye test, one was doubtful and negative, one was doubtful and positive, two were positive, and one was negative by the inhibition test. Out of 4 cases positive at 1:64 by the dye test, one was doubtful and three were positive by inhibition test. The single case positive at 1:256 by the dye test was also positive by inhibiton test. Out of 16 cases $<1:16$ by dye test, 8 were negative by duplicate inhibition tests, 2 were doubtful and negative, one was doubtful and positive, two were doubtful and 3 were positive. If one interprets a 1:16 dye test titer as

a borderline positive, and if one considers a positive or negative inhibition test coupled with a doubtful reaction in the duplicate test as being a borderline reaction, then the results obtained in group C and D can be summarized as follows: Out of 50 cases with reasonably satisfactory clinical histories, 27 were definitely positive by dye test, and 30 were definitely positive by the inhibition test; duplicate inhibition tests showed no serious differences and, in general, there was very good agreement between the dye and the inhibition tests.

The results obtained in groups C and D indicated that with these types of sera the inhibition test was reproducible to a high degree and its results paralleled those of the dye test. In order to compare the results of the inhibition test with those of the complement-fixation (C-F) test for toxoplasmosis, an additional group of 100 random sera (group E) was tested. For 30 of these, both dye and C-F test results[4] were available; the other 70 had been tested previously only by the C-F test. The results of the dye test, duplicate inhibition tests, and C-F tests on the first 30 sera are given in Table V. The parallelism of the inhibition and dye tests in this group is evident. Out of 15 sera $<1:16$ by dye test, 12 and 13 were negative or doubtful by duplicate inhibition tests, 3 and 2 sera, respectively, were positive by the inhibition method. Out of 6 sera positive by the dye test at the low titer of 1:16, 4 and 3 were positive by duplicate inhibition tests, 2 and 3, respectively, were negative or doubtful. Out of 9 sera positive by dye test at a titer 1:64 or higher, 8 and 7 were positive by inhibition tests, and the other 1 and 2 sera were negative or doubtful. On the other hand, the lack of correlation between the inhibition test and the C-F test was shown by the fact that the latter method detected only one positive in the group, compared to 12 or 15 positives detected by the inhibition method. A comparison of C-F and inhibition test results for the other 70 sera is shown in Table VI. Again, it is obvious that the inhibition test detected many more positives (24 to 1) than the C-F test. In the over-all group of 100 sera, there were 39 positives reported by one inhibition test, and 36 by the other; 5 cases which were negative in one inhibition test were positive in the duplicate test and there were 27 sera which were positive or negative in one test and doubtful in the duplicate run.

Inhibition Tests with Known Heterologous Antisera.—Up to this point, the specificity of the inhibition test for *Toxoplasma* antibodies was based on correlation with the dye test and clinical histories. Since questions have been raised concerning the specificity of the dye test (5), another approach was used to judge the kind of antibodies revealed by the inhibition test. Sera were

[4] Complement-fixation tests were performed by the Serology Laboratory of the Parasitology and Mycology Section, Communicable Disease Center, Atlanta, using antigen prepared from peritoneal exudate of infected mice.

TABLE IV

Relationship of Dye Test, Fluorescence Inhibition Test, and Clinical Histories in 50 Selected Sera

Serum No.	Reciprocal of dye test result	Fluorescence inhibition test 1	Fluorescence inhibition test 2	History
			Group C	
217	<16	Positive	Positive	13 yrs. old; bilateral chorioretinitis, fast deterioration of vision past 2 years, active lesions.
218	256	Positive	Positive	Mother of No. 217; no symptoms.
605	64	Positive	Doubtful	2 mos. old; chorioretinitis, convulsions, cerebral calcifications.
606	256	Positive	Positive	Mother of No. 605; no symptoms.
696	64	Positive	Positive	Repeat specimen on No. 605, 1 mo. later.
697	256	Positive	Positive	Repeat specimen on No. 606, 1 mo. later.
705	1024	Positive	Positive	Mother of baby which died with brain calcifications.
739	65,000	Positive	Positive	4 mos. old; chorioretinitis, hydrocephalus, cerebral calcifications, enlarged liver and spleen.
740	16,000	Positive	Positive	Mother of No. 739; no symptoms.
748	64	Positive	Doubtful	12 yrs. old; chorioretinitis, hydrochephalus of long standing.
749	256	Positive	Positive	Mother of No. 748; no symptoms.
771	64	Positive	Positive	11 yrs. old; old chorioretinitis.
772	256	Positive	Positive	Mother of No. 771; no symptoms.
776	64	Doubtful	Positive	Patient's infant had fever and hydrocephalus, no serology available on infant.
796	256	Positive	Positive	Repeat of No. 217, 6 mos. later.
797	256	Positive	Positive	Repeat of No. 218, 6 mos. later.
801	256	Positive	Positive	Mother of premature infant which died at 1 day with small brain lesions.
824	1024	Positive	Positive	19 yrs. old; active chorioretinitis.
826	1024	Positive	Positive	Mother of child with chorioretinitis; no serology on child.
836	1024	Positive	Positive	Mother of baby with high titer for toxoplasmosis.

TABLE IV—*Continued*

Serum No.	Reciprocal of dye test result	Fluorescence inhibition test 1	Fluorescence inhibition test 2	History
			Group C (Continued)	
866	256	Positive	Positive	Adult with brain lesions containing toxoplasma pseudocysts.
869	256	Positive	Positive	19 mos. old.
870	256	Positive	Positive	Mother of No. 869; no symptoms.
1084	1024	Positive	Positive	Repeat of No. 705, 4 mos. later.
1129	64	Positive	Positive	7 yrs. old; chorioretinitis, mentally retarded.
			Group D	
784	<16	Positive	Positive	16 mos. old; chorioretinitis, rash, psychomotor retardations, pneumonitis.
785	16	Positive	Positive	Mother of No. 784, no symptoms.
1047	64	Doubtful	Doubtful	10 months old; bilateral chorioretinitis, hydrocephalus.
1048	<16	Negative	Negative	Mother of No. 1047, no symptoms.
1051	<16	Positive	Positive	10 yrs. old; chorioretinitis, convulsions.
1052	<16	Positive	Positive	Mother of No. 1051, no symptoms.
1063	16	Negative	Doubtful	11 yrs. old; chorioretinitis, convulsions, cerebral calcifications, microcephaly.
1068	<16	Negative	Negative	8 mos. old; chorioretinitis, convulsions, psychomotor retardation.
1069	<16	Doubtful	Negative	Mother of No. 1068, no symptoms.
1071	<16	Negative	Negative	Newborn; chorioretinitis, microcephaly.
1072	<16	Negative	Negative	Mother of No. 1071, no symptoms.
1079	<16	Doubtful	Doubtful	13 yrs. old; chorioretinitis, psychomotor retardation, microcephaly.
1080	<16	Doubtful	Doubtful	Mother of No. 1079, no symptoms.
1089	<16	Doubtful	Positive	4 mos. old; chorioretinitis, psychomotor retardation.
1090	16	Positive	Doubtful	Mother of No. 1089, no symptoms.
1091	<16	Doubtful	Negative	4 yrs. old; chorioretinitis, nystagmus, retarded.
1092	<16	Negative	Negative	Mother of No. 1091, no symptoms.

TABLE IV—*Concluded*

Serum No.	Reciprocal of dye test result	Fluorescence inhibition test 1	Fluorescence inhibition test 2	History
			Group D (Continued)	
1096	<16	Negative	Negative	13 yrs. old; chorioretinitis, cerebral calcifications, hydrocephalus.
1107	256	Positive	Positive	Adult with chorioretinitis scars in each eye.
1118	<16	Negative	Negative	2 mos. old; chorioretinitis, convulsions.
1119	<16	Negative	Negative	Mother of No. 1118, no symptoms.
1130	16	Positive	Positive	7 yrs. old; chorioretinitis, fever.
1120	64	Positive	Positive	Mother of No. 1130, no symptoms.
1126	16	Negative	Negative	16 yrs. old; chorioretinitis, scars since 2 yrs. old, acute choroiditis.
1127	64	Positive	Positive	Mother of No. 1126, no symptoms.

TABLE V

Relationship of Dye, Fluorescence Inhibition and Complement-Fixation Tests on 30 Randomly Selected Sera

Group E

Test used	Test results	No. of sera at indicated dye test titer				No. of sera tested
		<16	1:16	1:64	1:256 and higher	
Dye test		15 (50)*	6 (20)	7 (23)	2 (7)	30
Fluorescence inhibition test 1	Negative	7 (23)	1 (3)	1 (3)	0	9
	Doubtful	5 (17)	1 (3)	0	0	6
	Positive	3 (10)	4 (13)	6 (20)	2 (7)	15
Fluorescence inhibition test 2	Negative	8 (27)	2 (7)	0	0	10
	Doubtful	5 (17)	1 (3)	1 (3)	1 (3)	8
	Positive	2 (7)	3 (10)	6 (20)	1 (3)	12
Complement-fixation test	Negative	14 (47)	6 (20)	5 (17)	1 (3)	26
	Doubtful	1 (3)	0	1 (3)	1 (3)	3
	Positive	0	0	1 (3)	0	1

* Figure in parenthesis is the percentage based on 30 sera tested.

obtained from 111 animals before and after immunization or infection with a variety of antigens. Another 11 postimmunization sera were obtained where preimmunization sera were not available. Each of the animals had been exposed to one of the following antigens: *Salmonella* sp., *Pasteurella pestis*,

Pasteurella pseudotuberculosis, *Streptococcus* sp., *Malleomyces mallei*, *Malleomyces pseudomallei*, *Bacterium tularense*, 9 serotypes of *Leptospira*, *Trichinella spiralis*, *Schistosoma mansoni*, *Trypanosoma gambiense*, *Entamoeba histolytica* and agents of lymphocytic choriomeningitis, vaccinia, St. Louis encephalitis, psittacosis, rabies, and typhus. Using standard serological procedures for the particular antigen, all postimmunization sera showed distinct and often high antibody titers to the homologous antigens. Inhibition tests with these sera showed no instances of positive reactions in postimmunization sera when the corresponding preimmunization serum was negative. Eighty two of the donor animals in this series were rabbits. One of these was doubtful before immunization with *P. pestis* and was positive 15 days later when the tube agglutination titer for the homologous antigen was 1:640. Another rabbit was positive by inhibition test both before and after immunization; the dye

TABLE VI

Relationship of Fluorescence Inhibition and Complement-Fixation Tests on 70 Randomly Selected Sera

Group E

Test	Results		
	Negative	Doubtful	Positive
Fluorescence inhibition test 1	33	13	24
Fluorescence inhibition test 2	28	18	24
Complement-fixation test	66*	3	1

* Includes one serum which was anti-complementary.

test titer for both sera turned out to be 1:1024. Out of 14 humans tested, 1 was positive by inhibition test both before and after immunization, the others were negative. The other donor animals in this series were distributed among the following species: mice, rats, hamsters, guinea pigs, goats, chickens, horses, cows, pigs, pigeons, turkeys, sheep, and monkeys. No definitely positive inhibition tests were obtained in any of these.

DISCUSSION

The phenomenon of inhibition of specific staining, whether by the sequence or combined method, seems to be readily explainable in terms of competition by labelled and non-labelled antibody molecules for available antigen sites. It has been shown that introduction of fluorescein into bovine serum albumin did not alter significantly the size, shape and homogeneity of the precursor molecules (9). If this is also true for antibody globulin, then in mixtures containing both labelled and non-labelled antibody, the degree of brightness of the antigen-antibody complex would depend upon the relative concentrations of the two types of antibody. It is not known why the combined method showed clear-cut inhibition with material that gave poor results by

the sequence method. This phenomenon has also been noted with bacteria (8), and therefore is not explainable entirely in terms of the species of organism involved.

The best reproducibility with the inhibition test was obtained in sera from cases selected on the basis of clinical histories (groups C and D). In these groups there were no reversals of positive and negative results in duplicate tests. In the groups chosen at random (B and E), greater variation was encountered so that there was a 3 to 4 per cent difference in the number of positives reported by the duplicate tests. On the basis of available information, one can only speculate on the reasons for the discrepancies between duplicate tests. Inhibition tests reported in this paper involved pipetting 0.02 ml. quantities of sera. It is possible that, in cases of sera with borderline amounts of antibody, small errors in pipetting might have had sufficient relative effect to change the results of the test. This viewpoint is supported by several facts: in most cases of reversals from positive to negative, inhibition was not very marked even when the result was positive; in all of the groups tested, the number of sera which changed from doubtful to negative or positive in the duplicate test was always considerably greater than those that changed from definitely positive to definitely negative; fewest reversals occurred in group C in which clinical histories and dye test results indicated the definite presence of antibody, whereas the greatest number of reversals occurred in a randomly selected group in which sera might be expected to contain varying amounts of antibody. In addition to the factor of pipetting, the subjective estimation of degree of fluorescence is undoubtedly a possible source of error particularly, again, in borderline cases. Readings of the same smears by two individuals showed slight variations which did not affect the distinctly positive and distinctly negative results, but which did affect the doubtful type of reaction. The use of a simple and objective method for determining brightness of fluorescence would be a great help but, as yet, only the method of using neutral density filters as described above has been tried. From a practical standpoint, if variation can be limited to borderline cases only, the results of the test could still be considered significant. If the test has validity in relation to infection with *Toxoplasma*, a change in successive sera, drawn at different times, from a borderline reaction to either definitely positive or definitely negative could still be interpreted.

In interpreting the significance of inhibition test results, it is proper to consider first the results of testing 122 sera containing antibody to antigens other than *Toxoplasma*. In this group of postimmunization sera there were no cases in which individuals changed from negative to positive following immunization or infection with non-*Toxoplasma* antigens, and only one case of a doubtful reaction becoming positive after immunization. Since conventional serological procedures had demonstrated the presence of consider-

able homologous antibody in the postimmunization sera in this group, it is reasonable to conclude that the inhibition test described here is not affected by the presence of many non-*Toxoplasma* antibodies. It would have been desirable to include in this series more animals immunized with protozoan antigens but these were not available at the time. The question of possible cross-reactions with such forms as *Sarcocystis*, *Trichomonas*, and *Leishmania* remains unanswered.

Since interpretation of the inhibition results has been based mostly on a comparison with the results of dye and complement-fixation tests and with clinical histories, a brief interpretation of the results of these latter tests may be helpful at this point.

Available evidence indicates that dye test antibodies appear relatively soon after infection, within a few weeks or less, whereas complement-fixing antibodies do not appear until later. In addition, dye test antibodies persist in diminishing titers for many years beyond the period of acute infection, whereas complement-fixing antibodies diminish sharply or disappear within 2 to 4 years. It follows from this that infants born with active infections may show distinct dye test titers but still be negative by complement-fixation. Ordinarily their mothers will have high titers in both tests (10). Serological evidence of infection with toxoplasmosis during a specific time period can be established by showing a rise in dye test titer, generally to a level of 1:256 or more; or if the dye test remains constant at high level, by showing a significant rise in complement-fixation titer. Consistently moderate dye test titers in the consistent absence of complement-fixing antibodies may be taken to indicate an old infection with toxoplasmosis; this condition is commonly found in adults. In infancy and childhood chorioretinopathy is an important sign for the selection of patients in whom toxoplasmosis is suspected, since, in the absence of chorioretinal lesions, serologic confirmation of infection is very low (11).

Table IV, which presents inhibition and dye test results on each of 50 sera tested, shows the close parallelism between results of the two tests. In the discrepancies which occurred it was impossible to say for sure which result was more accurate because further study of the cases concerned was not possible. However, some presumptive analyses are possible. In the case of No. 217, the positive result of the inhibition test is more in accord with presently accepted interpretations of toxoplasma serology than the $<1:16$ or "negative" result of the dye test. When this 13 year old child was retested 6 months later, (No. 796), both the dye and inhibition tests were positive. If the positive titer was related to the active chorioretinal lesions exhibited then the first serum (No. 217) should have been positive by dye test also, since there was evidence of visual deterioration for 2 years before the first serum was drawn.

The results of the dye test and inhibition test on paired sera 784–785, 1051–1052, 1089–1090, and 1126–1127 differ to some degree, but either set of results is compatible with accepted theory.

Serum 1047 was positive at 1:64 in the dye test, but was doubtful in the inhibition test. Since this was from a 10 month old infant whose mother, No. 1048, was negative by both tests, the positive dye test on the infant is unexpected. Clinically, there was reason to suspect congenital toxoplasmosis but serologically such a diagnosis could not be made unless the mother also showed a high dye test titer. The possibility exists that this was a case of acquired toxoplasmosis in the infant but there was no clinical evidence to support such a view. The simpler and more likely explanation is that the clinical symptoms were due to reasons other than toxoplasmosis, and that the dye test was in error in this case.

A greater number of discrepancies between the two tests occurred in groups B and E, in which sera were chosen at random. From Tables III and V it can be seen that most of the discrepancies occurred in sera with dye test titers of 1:16 and 1:64. From 61 to 72 per cent of the 1:16 dye test sera, and from 68 to 84 per cent of the 1:64 dye test sera were found positive or doubtful by the inhibition test, depending upon which of the duplicate inhibition results is used. Conversely, from 36 to 45 per cent of sera with dye test titers of $<$1:16 were positive by the inhibition test.

Causes for discrepancies between the two tests may be divided into inherent and technical reasons. Some obvious inherent reasons may be: (*a*) the tests measure different antigen-antibody systems which may or may not coincide in any one serum; (*b*) the antigen-antibody systems may be the same but the dye test is more sensitive and therefore picks up some positives that are missed by the inhibition test; (*c*) an anti-accessory factor substance may be present in some sera which may cause the dye test to appear negative even though antibody is present and detectable by the inhibition test; and (*d*) the remarkable sensitivity of the dye test may result in the detection at a low titer of non-specific antibodies which are non-reactive in the inhibition test. These possible reasons cannot be either confirmed or denied on the basis of available evidence.

Technical bases for discrepancies may be the variations which can occur in replicate tests of sera by both the dye and inhibition test. Since fourfold differences in the dye test titers of aliquots of the same serum are not unexpected (4), the number and identity of sera showing any given titer is likely to be different when a large enough group of randomly selected sera is tested more than once. In a group of 50 sera, aliquots of which were dye-tested as unknowns by four different, established laboratories, there were 26 cases of fourfold differences in titer, and seven cases of 16-fold differences among some

of the laboratories.[5] Most of the 1:16 and 1:64 dye test titers referred to in this paper are the results of single, screening tests. It might well be expected that in retesting such sera there would be shifts in the titers obtained in at least some of the cases. Under those circumstances the interpretation of some discrepancies between the dye and inhibition test results is well nigh impossible. Some of the technical sources of variation in the inhibition test have already been discussed in comparing the results of duplicate tests.

If the inhibition test is to serve as an adequate screening procedure for clinical diagnosis, to be followed up with the dye test, it should pick up as positive or doubtful those cases whose dye test titers are indicative of current or recent *Toxoplasma* infection. At present, authoritative interpretation of dye test results suggest that, in general, titers lower than 1:256 on single serum samples are difficult to relate to clinical disease, although in certain types of eye lesion cases the titer may be lowered to 1:64 (11, 12). In the work reported here, all sera with dye test titers of 1:256 or higher were positive or doubtful by the inhibition test; the same result was obtained in all sera from the clinically selected groups C and D which showed titers of 1:64. In the randomly selected groups B and E some 1:64 sera were negative by the inhibition test. Not enough sera have been tested to know whether this difference between the results obtained with group C and D and groups B and E is significant or not.

From the practical standpoint, the inhibition test has several advantages over the dye test as a routine procedure. Foremost is the fact that it uses a killed, standardized antigen which can be prepared in batches that are useful for about a month at a time. This contrasts with the dye test requirement of fresh live antigen obtained from infected mice immediately before use in the test. The dye test requires accessory factor, for which the only source at present is human serum. Since, in our experience, only about one in twenty young adults is a suitable donor, a reliable supply of this substance is a constant problem. The inhibition test does not require accessory factor. Reference has already been made to the variability of the dye test. Since the entire test must be set up and run before one can tell whether the controls are proper for the day, this variability results in the loss of much time. The relative simplicity of the mechanics of the inhibition test, and the use of previously titrated antigen and labelled antibody makes the routine performance of the inhibition test easier and more reliable.

The general procedures of the inhibition test are such that the test may be useful in the diagnosis of other conditions besides toxoplasmosis. The cyto-

[5] Unpublished data from the Toxoplasma Diagnostic Laboratory of the Communicable Disease Center, Atlanta, which, under the direction of Dr. M. M. Brooke, is acting as distributor of test sera to laboratories participating in this study.

chemical nature of the test means that only a relatively few cells or antigen particles are needed in order to test a serum, whereas large amounts are generally needed if one uses extraction methods to prepare soluble antigens. For example, in this laboratory, 1 ml. of mouse exudate serves to screen only about 15 sera by the complement-fixation test, but by the inhibition test about 200 sera can be screened with the same volume. This is not too significant in *Toxoplasma* testing because organisms are readily obtained in large numbers from infected mice, but it may be significant in working with agents like viruses or amebae.

There are important problems and difficulties associated with performing the inhibition test which need emphasis. A dark room, with the attendant problems of heating and cooling, is a necessity if the test is to be done with any sort of regularity. Fluorescence equipment is readily available commercially nowadays, but most technicians are not familiar with such equipment or with fluorescence methods and would need special training for the work. The subjective nature of reading test smears at present may influence the reproducibility of results from one laboratory to another. The preparation of labelled serum and *Toxoplasma* smears would probably be beyond the capabilities of most smaller laboratories. However, such material could be prepared centrally and be readily distributed to peripheral laboratories.

SUMMARY

A new serologic test for antibodies to *Toxoplasma* is described, which is based upon inhibition of specific staining with fluorescent antibody. In performing the test, a mixture of the test serum and known fluorescein-labelled antiserum is added to a dried smear of toxoplasms for 1 hour at 37°C. The smear is then rinsed and examined with a fluorescence microscope. Reduction in the brightness of fluorescence, as compared to that of a negative control slide, indicates the presence of antibody in the test serum.

A comparison of the results of this test with those of the methylene blue dye test showed a strong parallelism between the two sets of results. On the other hand, the complement-fixation test for toxoplasmosis did not yield nearly as many positives as the inhibition test.

The specificity of the new test was studied by comparing it with dye test results and clinical histories in human patients, and by testing a group of animals immunized with a variety of non-*Toxoplasma* antigens. No evidence of cross-reactions was obtained in the latter series.

Some advantages and disadvantages of the inhibition test are discussed.

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