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Dermal Hypersensitivity to Toxoplasma Antigens (Toxoplasmins).

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Both the toxoplasma-neutralizing antibody test[1] and the complement fixation test[2] have been used in patients to demonstrate specific antibodies to toxoplasma, indicative of past and possibly persistent infection. As pointed out by Sabin and Ruchman,[1] the neutralizing antibodies are unstable both at room and refrigerator temperatures, necessitating storage of test sera in the frozen state. Diagnostic readings require a week to develop. Equivocal results are frequently obtained, presumably due to low titers of neutralizing antibodies.[3] For performance of the test an active strain of toxoplasma must be maintained in the laboratory. While the toxoplasma-neutralizing antibodies seem to persist for a matter of years in the experimentally infected animal, complement-fixing antibodies appear to be more evanescent.[2] Many human cases giving a positive neutralizing-antibody test have been encountered where the complement fixation test has been negative. The presence of complement-fixing antibodies may be more indicative of a recent infection or of recent contact with toxoplasma antigen.

It appeared desirable, therefore, to investigate the possibility of developing toxoplasma antigens for skin tests which would be simple, rapid and less costly in performance. The preparation of several antigens will be described and preliminary data on their use in patients are given.

Experimental: (a) *Chick-embryo antigens*: It was felt originally that toxoplasma grown in the developing chick might give useful antigens, at the same time almost excluding the possibility of accidental infection of the host with other microorganisms. Sabin's RH strain[4] of toxoplasma was inoculated onto the freshly collapsed chorioallantoic membrane of the 7 to 9 day old chick embryo. These membranes were harvested 4 or 5 days after inoculation, which was about a day before the expected death of the embryo. The membranes were ground with alundum. The pulp was diluted with normal saline to one-tenth its concentration, then frozen in a carbon dioxide ice-alcohol mixture and thawed, repeatedly. The resulting material was centrifuged and the sediment discarded. The supernatant fluid was preserved with phenol 1:1000 or merthiolate 1:10,000 and then passed through a Seitz filter. Uninfected membranes were treated similarly and the product was used as control antigen.

Such filtered supernatant fluids were tested for potency in about 70 guinea pigs which had a chronic latent infection with toxoplasma. It was found that 1/10 cc of a 1 to 50 dilution of the test antigen would produce areas of erythema and induration of about 30 and 15 mm, respectively, after 24 hours, in infected animals, but erythema of not more than a few millimeters in normal control guinea pigs. Permitting the uncentrifuged membrane suspension to autolyse in the refrigerator for a few days slightly increased the titre of the test antigen. The control antigen gave rise to less than 5 mm of erythema in both normal and immune guinea pigs.

Chick-embryo toxoplasma and control antigens were employed in skin testing of patients during 1945-1947. Again 15+ mm of induration at the site of the test dose with not more than 4 mm of induration at the site of the control injection was considered a positive test. Most of the positive tests were apparent after 24 hours, with occasional reactions tak-

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1 Sabin, A. B., and Ruchman, I., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 1.

2 Warren, J., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 11.

3 Heidelman, J. M., *Arch. Ophth.*, 1945, **34**, 28.

4 Sabin, A. B., *J. A. M. A.*, 1941, **116**, 801.

ing 48 hours to develop. The latter time was therefore used routinely to read the tests. A positive test persisted usually for 4 to 10 days, while the erythema of the negative and the control tests, in most cases, had disappeared in 24 hours.

(b) *Hamster and Mouse Antigens*: Antigens from the sediment of peritoneal exudate of heavily infected hamsters and mice were developed in 1947 and largely replaced the chick-embryo antigen. In infected chick embryos toxoplasma were far in the minority, compared to the number of host cells as shown on sections through the chorioallantoic membranes. Consequently antigens prepared from chorioallantoic membranes contained a disproportionately large amount of chick embryo protein. Exudates of mice and hamsters 4 or 5 days after intraperitoneal infection with the RH strain of toxoplasma, contained leukocytes and organisms in about equal proportions. Toxoplasma were estimated to make up approximately one quarter of the volume of peritoneal exudate sediments. The latter gave positive skin reactions in infected animals and in patients in dilutions of 1:500 to 1:10,000. Hence, these antigens were of greater potency and they were associated with less carrier-protein.

Toxoplasma antigens derived from mouse peritoneal exudates are preferable to those from hamsters since the average yield of fluid and of organisms is greater from adult mice than from adult hamsters. The mice to be used for antigen preparation must be known to be free from paratyphoid and other pathogens.

Preparation of Toxoplasmin from Peritoneal Exudates. Mice (and similarly, hamsters) are injected intraperitoneally with 0.2 cc of a 1:50 dilution of peritoneal fluid from a mouse which had been infected intraperitoneally with the RH strain of toxoplasma. Four or 5 days after injection their abdomen becomes distended with peritoneal fluid. Anticipating their expected death from toxoplasmosis, mice are lightly etherized and the peritoneal fluid is aspirated through a previously disinfected area with a No. 20 needle fitted to a 5 cc syringe. The fluids obtained, which are rich in fibrinogen, are pooled in previously weighed centrifuge tubes containing enough heparin to make a final concentration of about 10 mg%. After thorough mixing the pooled fluids are centrifuged for 20 minutes at about 2000 RPM. The supernatant fluid is discarded since it is poor in antigenic material relative to its protein content. Then the tube is reweighed to determine the amount of sediment remaining. The latter corresponds to approximately 1% of the original fluid weight. The sediment is resuspended in 10 times its weight of saline. The suspension is then exposed in a watch glass to a General Electric germicidal lamp of 15W at a distance of 30 cm for 4 minutes or to an equivalent amount of ultra violet germicidal energy. The depth of the suspension should not exceed 2-3 mm. Both toxoplasma and accidental contaminants will be killed by this treatment. The 1:10 suspension is frozen rapidly in a carbon dioxide ice-alcohol mixture and thawed alternately for 5-10 times to break up the organisms. Subsequently a 1:1000 dilution is prepared with normal saline containing 0.25% phenol as preservative. Its protein content is in the neighborhood of 2.2 mg%. An injection of 0.1 cc of a 1:1000 dilution of mouse toxoplasmin contains approximately 8,000 autolysed organisms.

For the preparation of the control antigen mouse spleen appeared preferable to sterile peritoneal exudates produced by irritants. The latter were difficult to produce in amounts necessary to recover sufficient cell sediment. The complete disappearance time of the irritant injected was hard to determine with accuracy. In contrast to this, ground spleen suspensions are easily prepared. Their cell constituents are not vastly different from those of the peritoneal exudate after infection, which contains about 40% of macrophages, 30% of monocytes and lymphocytes and 30% of heterophile granulocytes, and variable amounts of red blood cells apart from toxoplasma. Hence the control antigen is prepared from ground normal mouse spleen and the pulp is treated like peritoneal fluid sediment.

(c) *Assays of the Antigens.* The antigens

are subjected to tests for non-toxicity and sterility including aerobic and anaerobic cultures, and the intracerebral inoculation of hamsters and mice. These animals should succumb to a challenge injection performed 3 weeks later. Seitz-filtration was not used since it removes much antigenic material.

Preliminary potency tests are performed on guinea pigs having chronic latent toxoplasmosis. Later the material is checked on known toxoplasmin reactors, also known to have neutralizing antibodies to toxoplasma and on non-immune human beings. Criteria for reading of the tests are similar for chick embryo, mouse or hamster antigens. It should be remarked here, that guinea pigs may quickly acquire dermal hypersensitivity to the carrier-protein, so that they should not be used for more than 2 or 3 skin tests. Human beings have not been observed to acquire dermal hypersensitivity to chick embryo, mouse, hamster or toxoplasma proteins, in result of repeated skin testing. Some infected guinea pigs do not seem to be hypersensitive to toxoplasmin. Most patients show more violent reactions to toxoplasmin than guinea pigs. For these reasons clinical assay of the antigens is necessary. A 1:1000 or 1:10,000 dilution of peritoneal exudate sediment was found to be a satisfactory antigen for use in patients. A test was considered positive if 10-30 mm of induration and 10-50 mm of erythema were present after 48 hours. The control injection rarely resulted in more than 3 mm of erythema at the end of 48 hours. Certain laboratory workers exhibited larger dermal reactions to injections of the control antigen, which appeared more rapidly and disappeared more quickly than a positive toxoplasmin test. Positive controls were more frequently encountered after the use of chick embryo than after employment of mouse antigens. Furthermore, mouse antigens gave rise to more marked quantitative differences between test and control antigen injections that were positive.

Antigenic potency was retained for periods of from 5 to 16 months, at which time all the material had been used. Antigen suspensions increased in potency upon storage, suggesting further disintegration of the previously broken-up toxoplasma into a more soluble protein.

(d) *Correlation of Dermal Hypersensitivity with the Presence of Neutralizing Antibodies in Patients.* Neutralizing antibodies are demonstrated by their inhibiting effects in toxoplasma-serum mixtures upon the development of papulonecrotic skin lesions in rabbits, at the sites of intradermal injection of various dilutions of toxoplasma. Neutralizing antibody tests were conducted essentially as described by Sabin and Ruchman.[1] The following modifications were used: 1. Peritoneal exudate of an intraperitoneally infected mouse was used instead of infected brain suspension. 2. The peritoneal exudate, containing many toxoplasma organisms, was diluted in normal saline rather than in Tyrode's solution. 3. Eight instead of 4 tests were performed on one rabbit. 4. Instead of tracing the lesions for the final reading, the rabbit skin was spread out on a glass plate and read by transillumination. This type of reading not only takes into account the linear extensions of the inflammatory process but also evaluates the intensity of the inflammatory lesion in depth. Each test skin bearing 8 separate tests was photographed by transmitted light and then fixed in formalin.

Patients who were skin tested repeatedly with negative results did not develop toxoplasma neutralizing antibodies. However, patients in whom a positive skin reaction was elicited, frequently did show an anamnestic rise in neutralizing antibody titre.

(e) *Correlation of Dermal Hypersensitivity with the Presence of Complement-Fixing Antibodies in Patients.* Duplicate specimens of sera investigated for neutralizing antibodies were tested for the presence of complement-fixing antibodies. These tests were conducted by Dr. Carl M. Eklund and Dr. David Lackman of the Rocky Mountain Laboratory, Hamilton, Montana, and will be reported at a later date. Two of their findings, however, should be quoted here. In the first place, patients who were skin tested repeatedly with negative results being obtained did not develop complement-fixing

TABLE I.
Comparison of Results Obtained by the Use of Toxoplasmin Skin Tests and Toxoplasma Neutralizing Antibody Tests in 108 Patients.

Neutralizing antibody test		Skin test		
		Positive	Negative	Equivocal
Positive	47	44	3	0
Negative	43	3	40	0
Equivocal	18	12	6	0
Total	108	59	49	0

TABLE II.
Percentage Correlation Between Toxoplasma Neutralizing Antibody Tests and Toxoplasmin Skin Tests Based on Data Incorporated in Table I.

Neutralizing antibody test		Skin test		
		Positive	Negative	Equivocal
	%	%	%	
Positive	100	94	6	
Negative	100	7	93	
Equivocal	100	67	33	

TABLE III.
Percentage Correlation Between Toxoplasmin Sensitivity and Results of Toxoplasma Neutralizing Antibody Tests Based on Data Presented in Table I.

Neutralizing antibody test	Skin test		
	Positive	Negative	Equivocal
	%	%	
Positive	75	6	
Negative	5	82	
Equivocal	20	12	
Total	100	100	

antibodies to toxoplasma antigen. Secondly, a positive toxoplasmin skin test did give rise to complement-fixing antibodies in a great majority of my patients so investigated.

Results. (a) *Comparison of data obtained by use of toxoplasmin skin tests and neutralizing antibody tests.* Table I presents the results obtained by the 2 tests in 108 patients. Table II indicates that a high percentage of analogous results are obtained with skin test, if the results of the neutralizing antibody tests are clearly positive or negative. Two thirds of the equivocally neutralizing sera were accompanied by positive skin tests and one third by negative ones. Table III illustrates in toxoplasmin positive and toxoplasmin negative patients, the scattering of results obtained with the neutralizing antibody test.

The sera of 3 toxoplasmin positive patients did not show toxoplasma neutralizing antibodies at the time of the initial skin test. However, neutralizing antibodies were found upon retesting with the identical serum specimen in one and with the second specimen in 2 of the patients. Furthermore, positive skin tests were obtained with skin testing material derived from chick embryo and hamster or mouse in each of the 3 patients. Hence these 3 negative neutralizing antibody tests must be considered to have been "falsely negative" ones. Two of the patients presented themselves with lesions of chorioretinitis, while a third one did not exhibit clinical signs suggestive of toxoplasmosis.

The 3 toxoplasmin negative patients whose sera contained toxoplasma neutralizing antibodies were not available for restudy. It is possible that their skin tests became positive after the 2 day observation period. A late appearance of signs of dermal hypersensitivity was observed in an occasional patient. None of the 3 patients showed clinical signs suggestive of toxoplasmosis.

The occurrence of an appreciable percentage of equivocal results is an objectionable feature of the neutralizing antibody test. Since a skin test acts as a booster injection, the rise in neutralizing and complement-fixing antibody titres following it, served as convenient evidence in favor of the specificity of an associated positive skin test. No such anamnestic rise has been noted, associated with a negative skin test. Hence most equivocally neutralizing antibodies appear to be due to low-titre neutralizing antibodies. Six of the 12 toxoplasmin positive patients either showed clinical signs suggestive of toxoplasmosis or were parents of toxoplasmic infants. None of the 6 toxoplasmin negative patients belonged in either of these groups.

(b) *Toxoplasmin sensitivity in certain selected and unselected individuals (Table IV).* The incidence of toxoplasmin sensitivity in a group of younger adults (medical students, laboratory and office workers), aged 20-35 years, was 10%. In an older group of

TABLE IV.
Toxoplasmin Sensitivity in Certain Selected and Unselected Individuals.

Grouping	Years of age	No.	Toxoplasmin- Positive	%	Negative	%	Equivocal	%
1. Survey—unselected		100	19*	19	81	81		
1a. " "	20-35	50	5	10	45	90		
1b. " "	50-83	50	14	28	36	72		
2. Patients with chorioretinitis†	Mean 23	28	20	71	8	29		
2a. Mothers of toxoplasmin positive patients		8	8	100				
2b. Mothers of toxoplasmin negative patients		2	1	50	1	50		
3. Patients with anterior uveitis†	Mean 35	40	13	33	27	67		
4. Infants with congenital toxoplasmosis, fatal, toxoplasma isolated	5 weeks	2			1	50	1	50
5. Infants with neonatal toxoplasmosis, surviving	Mean 2	2	2	100				
6. Mothers of No. 4, 5, and others	" 30	6	6	100				
7. Fathers of No. 4, 5, and others	" 30	4	2	50	2	50		
8. Siblings of No. 4, 5, and others	" 6	7			7	100		
9. Pathologists	" 36	7	4	57	3	43		
10. Patients with hydrocephalus without chorioretinitis	" 1	10	1	10	9	90		

* The first numeral refers to the number of toxoplasmin positive patients, the second to the percentage within the total group.

† Patients seen in conjunction with a study supported by the Francis I. Proctor Foundation for Research in Ophthalmology of the University of California.

hospital patients selected at random, most of which were affected with neoplasms or heart disease, a 28% incidence of toxoplasmin sensitivity was demonstrated.

The high incidence of positive toxoplasmin reactors in patients with chorioretinitis is of especial interest. The mean age of these toxoplasmin positive patients when first seen was 23 years. The mean age when their disease was first noted was 13 years. For the toxoplasmin negative group of patients with chorioretinitis both dates were near 28 years of age. Both patients in Group 5 had chorioretinitis and nystagmus soon after birth. Only one of them, however, is markedly retarded mentally, and he has cerebral calcifications.

Groups 6, 7 and 8 refer to family members of 4 patients with toxoplasmosis proven by pathological examination at autopsy. From 2 patients the organism was also isolated (Group 4). However, the diagnosis in cases of Group 5 rested on clinical evidence, corroborated by serological data. The patient in Group 10, who was toxoplasmin positive, was 10 years of age. He did not show other clinical signs of toxoplasmosis.

(c) *Demonstration of toxoplasma antigen in ventricular fluid of infants with hydrocephalus.* Fluids obtained from ventricular puncture were centrifuged. One-tenth cm^3 of the supernatant was injected intradermally into normal guinea pigs and into guinea pigs with chronic latent toxoplasmosis. Ventricular fluid from 2 patients from whom toxoplasma was isolated, evoked after 24 hours a large area of erythema and induration in the infected guinea pigs. No reaction was present in the normal animals. Fluid from a patient with *Escherichia coli* meningitis evoked a slight area of erythema and induration in the toxoplasmic guinea pigs, but none in the normal controls. Ventricular fluids from 4 other hydrocephalic infants gave negative reactions in both groups of guinea pigs. Guinea pigs previously injected with human brain or cerebrospinal fluid may not be used, since they show marked reactions of dermal hypersensitivity on reinjection with such material.

Discussion. The high degree of specificity of the toxoplasmin reaction was initially ascertained in normal guinea pigs and in those with chronic, latent toxoplasmosis. These findings were supported by a high degree of correlation between skin hypersensitivity and

the presence of toxoplasma neutralizing antibodies in man. This specificity was further confirmed by the anamnestic appearance or rise of complement-fixing and neutralizing antibodies, following injection of the skin test antigen into individuals exhibiting dermal hypersensitivity. The high incidence of toxoplasmin sensitivity in patients with chorioretinitis, with and without associated cerebral calcifications, and in the mothers of such patients is significant.

The ease of performance of the skin test and the rapidity with which diagnostic readings appear make this test most useful in surveys. Equivocal results which are so frequently obtained with toxoplasma neutralizing antibody tests[3] were of exceedingly rare occurrence with the use of the skin test. False positive skin tests were not encountered. The incidence of falsely negative skin tests was possibly 3%, however, the chance that neutralizing antibody test might have been falsely positive on some of these patients cannot be excluded at present. Hence the importance of performing on the same patient skin tests, neutralizing antibody tests, complement fixation tests and the new tissue culture tests with the patient's white blood cells,[5] in the presence of toxoplasma antigen. Reading the skin test after 96 as well as 48 hours might substantially reduce the number of false negative skin tests.

New light has been shed on the incidence of asymptomatic past and possibly persistent toxoplasmosis in the general population. Whereas there may be differences in various regions of the country, the relatively low figures quoted previously[6,7,3] might be largely due to the use of the neutralizing antibody test, which is less sensitive than the skin test. The differences in age incidence point to the common occurrence of asymptomatic infection in adults. The incidence of toxoplasmin sensitivity in patients with chorioretinitis, observed to be 71% in my patients, is higher than previously reported.[3,8] Toxoplasmin has been used in the treatment of active chorioretinitis, which was associated with skin test and serological evidence of toxoplasmosis. This will be discussed in another publication.

The use of ventricular fluid of infants with hydrocephalus is suggested as an aid in making or disproving a presumptive diagnosis of neonatal toxoplasmic encephalitis.

Summary. The preparation of toxoplasmin, a skin testing antigen made of toxoplasma has been described. Toxoplasmin evokes reactions of a delayed (tuberculin) type of hypersensitivity in certain patients. There is a high correlation between toxoplasmin hypersensitivity and the presence of toxoplasma neutralizing antibodies. The latter may be of very low titre and may not be unequivocally demonstrable until an anamnestic rise has occurred following the injection of the skin test dose. The ease of performance and the rapidity with which clear-cut results are obtained, make the toxoplasmin skin test the most useful single aid in the diagnosis of past or latent toxoplasmosis, wherever isolation of the caustive organism is not feasible. A test based on dermal hypersensitivity has been described facilitating the differential diagnosis of neonatal hydrocephalus.

5 Nantz, F. A., and Blatt, H., *Ann. Allergy*, 1947, **5**, 554.

6 Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 6.

7 Callahan, W. P., Jr., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 68.

8 Johnson, L. V., *Arch. Ophth.*, 1946, **36**, 677.