The incidence of toxoplasmosis in a mentally retarded population
by Marlene Joyce Mackie

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Microbiology
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Abstract:
Serums from 478 mental defectives from the Boulder River School and Hospital in Boulder, Montana were tested for antibodies for Toxoplasma gondii using the Indirect Fluorescent Antibody Test (IFAT) and the Indirect Hemagglutination Test (IHA). Serums for a control population consisting of specimens for Billings and Helena, Montana were tested concurrently. All populations were separated according to age and sex. In the control populations, the IFAT showed an increase in the percent of serums positive from ages 20-40 years. The age groups from 30-50 years showed a high percent positive in the IHA test. The Boulder group showed no rise in incidence until age 50 years in the IFAT while the IHA test showed a more even distribution in all age groups. In all three groups, the percent of serums positive for each sex was essentially no different. Agreement between the IFAT and the IHA1test was 38 percent in Helena, 43 percent in Billings, and 36 percent in Boulder. In the mentally defective group, the patients positive by the IFAT or IHA test were separated according to time in residence at the institution and type of mental retardation. The results showed little variation of the percent of positive serums in the length of time in residence or in the type mental retardation. Elevation and yearly rainfall statistics for Boulder, Helena and Billings, Montana were compared with the percent positive serums in these areas. The results showed essentially no difference.

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Date August 4, 1970

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MARLENE JOYCE MACKIE

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

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Approved:

William G. Walter
Head, Major Department

Alvin D. Hiscox
Chairman, Examining Committee

R. L. Goering
Graduate Dean

MONTANA STATE UNIVERSITY
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Serums from 478 mental defectives from the Boulder River School and Hospital in Boulder, Montana were tested for antibodies for Toxoplasma gondii using the Indirect Fluorescent Antibody Test (IFAT) and the Indirect Hemagglutination Test (IHA). Serums for a control population consisting of specimens for Billings and Helena, Montana were tested concurrently. All populations were separated according to age and sex. In the control populations, the IFAT showed an increase in the percent of serums positive from ages 20-40 years. The age groups from 30-50 years showed a high percent positive in the IHA test. The Boulder group showed no rise in incidence until age 50 years in the IFAT while the IHA test showed a more even distribution in all age groups. In all three groups, the percent of serums positive for each sex was essentially no different. Agreement between the IFAT and the IHA test was 38 percent in Helena, 43 percent in Billings, and 36 percent in Boulder. In the mentally defective group, the patients positive by the IFAT or IHA test were separated according to time in residence at the institution and type of mental retardation. The results showed little variation of the percent of positive serums in the length of time in residence or in the type mental retardation. Elevation and yearly rainfall statistics for Boulder, Helena and Billings, Montana were compared with the percent positive serums in these areas. The results showed essentially no difference.

INTRODUCTION

In 1908, Toxoplasma gondii was isolated by two different groups working independently. Nicolle and Manceaux (1908) isolated the organism from Ctenodactylus gondii, a rodent in Africa and Splendore (1908) isolated it from rabbits in Brazil. In 1908, Darling isolated cyst forms from the arm muscle of a Barbadian negro. From 1908 to 1939, data were published which dealt primarily with morphological descriptions and the occasional occurrences in various animals and man (Feldman, 1968a). Wolf et al. (1939) demonstrated that the organism invaded the fetus in utero resulting in a stillborn or diseased infant. Two years later, acquired toxoplasmosis was described in children as an encephalitis by Sabin and in adults as a spotted fever syndrome by Pinkerton and Henderson (1941). Since that time, numerous reports citing toxoplasmosis as a multisystem disease (Altman, 1968) have emanated from North and South America, Europe, Africa, Asia, and Oceania (Wright, 1957).

Toxoplasma is an obligate, intracellular parasite (Sabin and Olitsky, 1937) able to infect a wide range of animal hosts including marsupials, moles, rodents, carnivores (Moller, 1952), swine (Farrell et al., 1952), sheep, cattle (Jacobs, 1960b), various simians (Sabin and Ruchman, 1942; Cowen and Wolf, 1945), birds (Jacobs and Jones, 1950; Jacobs et al., 1952) and some reptiles (Frenkel, 1953; Jacobs, 1956).

It proliferates in all cells in the vertebrate host with the exception of the non-nucleated red blood cells (Feldman, 1968). Entry into the cell is facilitated by the use of lysozome-like structures and hyaluronidase (Norby et al., 1968; Lycke et al., 1965). Once inside the host cell, Toxoplasma is capable of competing with the host cell for nutrients and metabolites (Jacobs, 1963). Eventually, this process results in host cell rupture with the resultant spread of virulent organisms to other cells (Jacobs, 1963). A variation of the virulent life cycle often occurs when cysts develop in a vacuole of the host cell (Jacobs, 1967). As this occurs, the proliferation of the organism subsides, the symptoms of the disease disappear, leaving the host with chronic toxoplasmosis for life. The condition does not incapacitate the host but symptoms may recur when a cyst ruptures releasing viable organisms which are able to reinfect susceptible cells (Jacobs, 1963). More recent evidence indicates that the chronic state may not be induced by cyst rupture only, but by the persistence of residual trophozoites intracellularly without host cell rupture (Jacobs, 1967).

In summary, the characteristics of Toxoplasma which are crucial in its' pathogenicity are: (1) its' ability to grow in many types of cells; (2) its' ability to form a cyst; and (3) its' obligate, intracellular development (Altman, 1968).

Early studies on transmission of Toxoplasma gondii centered about the trophozoite. The organism was found to be fragile, not resistant to osmotic pressure changes, and incapable of surviving for long periods extracellularly (Jacobs, 1968). Since the acute disease is characterized by a parasitemia, investigations were directed toward blood sucking arthropods as reservoirs of the disease. Mosquitoes, fleas, triatomid bugs, bedbugs, lice, mites, and ticks were tested with no success (Jacobs, 1968).

Large numbers of trophozoites introduced directly into the stomach of the recipient host failed to produce the disease while the feeding of tissue from chronically infected animals did (Jacobs, 1968). This initiated studies which isolated the cyst from animal tissue, showed its' incidence and characterized its' resistant qualities (Jacobs et al., 1960a; Work, 1968). Data published by Jacobs et al. (1960a) and Work (1968) showed that the cyst is resistant to gastric juices for 2-3 hours, resistant to freezing, but susceptible to heat. They also showed that the viable trophozoites released from a cyst are capable of surviving in an environment simulating the small intestine, firmly establishing the feasibility of the oral route of transmission through undercooked meat (Kean et al., 1969). Although this offered a partial explanation, additional information was needed since many animals and human populations

which are strict herbivores (Jacobs et al., 1954b; Jacobs et al., 1960b) have a high incidence of toxoplasmosis. Research initiated in 1968 by Hutchinson et al. linked transmission of the organism with the egg of the nematode, Toxocara cati, in the feces of an infected cat. However, further studies showed that it is possible to transmit Toxoplasma in the absence of a nematode infection (Frenkel et al., 1969; Sheffield and Melton, 1969). Work and Hutchinson (1969) have since isolated what appears to be a new cyst form, from fecal floats of infected cats, which is able to produce the disease when inoculated into laboratory mice intraperitoneally. The oral route of transmission could explain the widespread incidence in populations with poor sanitation standards (Remington, 1970).

In addition to the modes of transmission discussed above, congenital transmission via the placenta has long been established in humans and animals (Wolf et al., 1939; Remington et al., 1961; Jacobs, 1968).

The syndromes caused by Toxoplasma gondii are extremely varied but can be placed into two major groups, congenital and acquired.

Congenital toxoplasmosis occurs as an accidental complication of an inapparent primary infection of a pregnant human (Feldman and Miller, 1956b). The manifestations in the infant depend on the virulence of the organism, the resistance of the mother, and the

stage of pregnancy (Feldman and Miller, 1956b). If the infection occurs in the first trimester of pregnancy, fetal death is most common; if it occurs during the second trimester, the result is widespread disease causing infant death or such severe conditions as hydrocephalus, microcephalia, psychomotor retardation, cerebral calcifications, chorioretintis or microphtalmus. Infections occurring during the last trimester produce acute symptoms of encephalitis with fever, generalized convulsions, and various paralyses and viceral involvement in the form of jaundice, anemia, purpura, petichiae, lymphadenopathy, hepatosplenomegaly, and chorioretinitis (Altman, 1968). The pattern of congenital toxoplasmosis is outlined in Figure 1 (Macer, 1963).

Of the infants infected congenitally, one-fourth are premature with a mortality rate of 27 percent. Of those babies carried to term, 12 percent die and of those that survive, 80 percent are defective (Altman, 1968). Feldman (1953) found psychomotor retardation in 56 percent, hydrocephalus or microcephalia is 33 percent each, convulsive disorders in the first three years in 50 percent, chorioretinitis in 80-90 percent with bilateral involvement in 85 percent, and cerebral calcifications in 63 percent of youngsters who tested Methylene Blue Dye test positive (Altman, 1968; Sabin and Feldman, 1948). It is important to note that often the only evidence

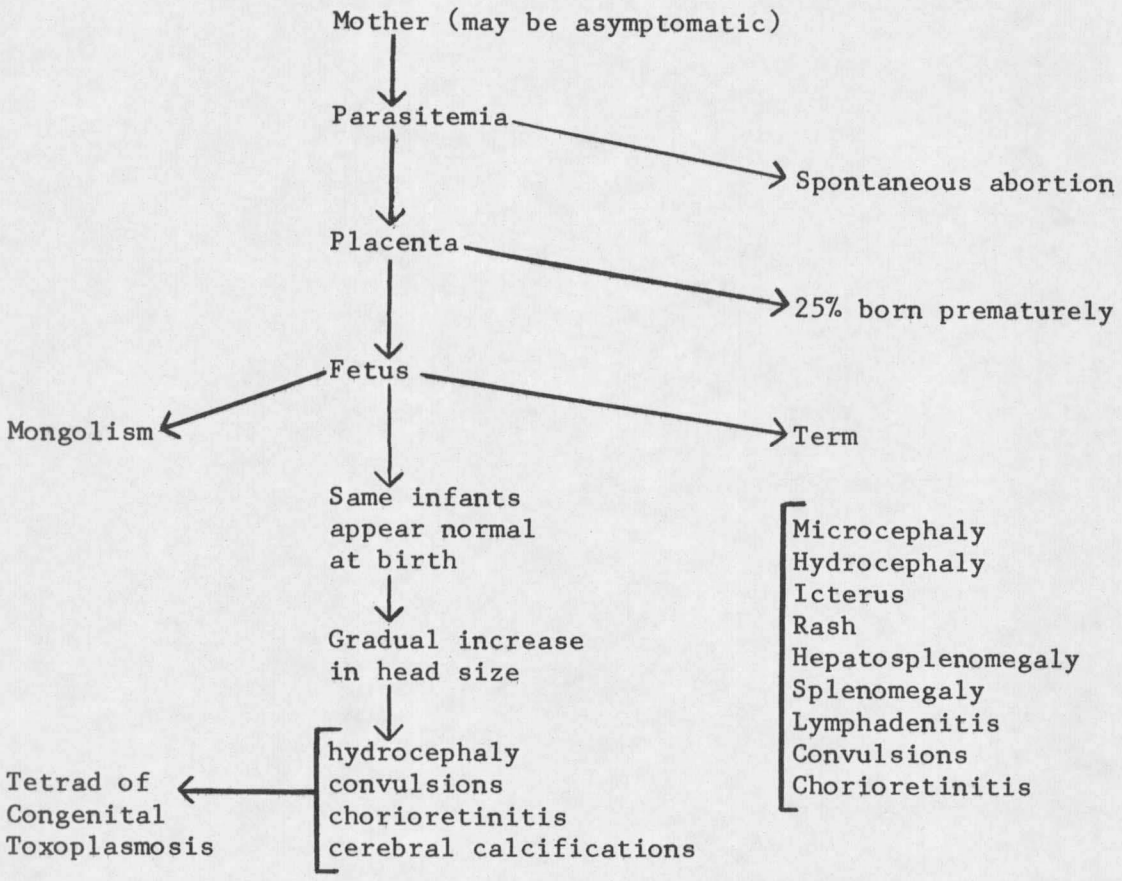

Figure 1. Pattern of congenital toxoplasmosis.

of congenital toxoplasmosis is the ocular involvement and that the acute, fulminating disease producing the classic tetrad of symptoms including chorioretinitis, hydrocephaly or microcephalia, psychomotor retardation and cerebral calcifications is much less common than the subacute asymptomatic form (Fair, 1959; Franke, 1960).

Feldman and Miller (1956) generalized the following about congenital toxoplasmosis: (1) transmission can take place in any season; (2) a recent infection is usually observed in the mother but rarely in the father; (3) sex of the fetus was not a factor; (4) twins are usually both infected, but not to the same extent; (5) mothers who have antibodies before conception do not produce congenitally infected children; (7) not all intrapregnancy infections lead to fetal disease and some fetuses escape infection altogether; and (8) subsequent children are not congenitally infected. The last generalization has been disputed by researchers who feel that his may not always be the case. They stress that chronic toxoplasmosis is an important cause of repeated spontaneous abortions, stillbirths, and premature deliveries (Feldman, 1968b).

Acquired toxoplasmosis presents an even more diverse symptomologic picture. The four manifestations of the disease are: (1) lymphodonosa, (2) exanthematica, (3) cerebrospinalis, and (4) opthalmica (Siim, 1956). The most common is the lymphatic form. The

onset may be acute or subacute and the course may be febrile, afebrile, or subclinical (Feldman, 1968b). Non-specific symptoms such as headaches, nausea, abdominal pain, arthralgias and myalgias may be present during the course of the disease (Feldman, 1968b). All nodes, including hilar, mesenteric, and retroperitoneal may be involved. Biopsy of the node reveals typical forms of Toxoplasma which when inoculated into laboratory animals will produce the c. disease (Siim, 1956; Feldman, 1968b). This form of the disease may mimic many other diseases such as infectious mononucleosis (Remington et al., 1962), glandular fever (Siim, 1956), Hodgkin's disease (Jacobs, 1967) and lymphadenopathy of unknown origin (Siim, 1956) and for this reason is often misdiagnosed.

Immunity against the organism consists of macroglobulin (19S) which occurs in both the acute congenital or acquired syndromes. 7S antibodies are demonstrated only in infants born to chronically infected mothers (Remington et al., 1966). The macroglobulins provide the basis for many of the serologic tests presently used in diagnosis of the disease.

The Methylene Blue Dye test developed by Sabin and Feldman in 1948 was the first serologic tool for the diagnosis of toxoplasmosis. However, the inconvenience of dealing with live parasites and the difficulty in obtaining large amounts of the "accessory" factor,

found only in fresh human serum, initiated a search for new methods of testing.

A complement fixation procedure was developed in 1954 by Awad (Eichenwald, 1956). The test was not a useful diagnostic tool because antibody titers did not rise until late in the course of the disease and receded after a short time, making it difficult to obtain an early diagnosis.

A hemagglutination test was developed in 1957a by Jacobs and Lunde. The procedure showed promise because of the stability of the antigen and the rapid reading of the test results (Jacobs and Lunde, 1957a). Additional adaptation to microtechniques has further simplified it by increasing the rapidity of performance and decreasing the reagent demand (Lewis and Kessel, 1961).

Other tests have been described including a direct agglutination test (Fulton and Turk, 1959) and a flocculation test using acrylic particles (Siim and Lind, 1960), however, these have not been used frequently.

In 1959, Carver and Goldman reported a method of staining Toxoplasma gondii present in tissue sections with fluorescein labeled antibody. This work led directly to the development of the Indirect Fluorescent Antibody test (Kelen et al., 1962). The procedure was further developed and the specificity validated by Fletcher in 1965,

Sulzer and Hall in 1967, and Chessum in 1970. They found that the test was as sensitive as the Methylene Blue Dye test but more desirable because it had a sharpened point, stable reagents which were easily prepared and standardized, and there was no need for live parasites or the "accessory" factor.

A skin test using a water-lysed suspension of organisms relies on the production of a delayed type of hypersensitivity (Jacobs, 1963). It is used primarily in epidemiological studies where large numbers of people are tested.

Finally, toxoplasmosis can be diagnosed through isolation of the organisms from the tissues (Walls et al., 1963) and inoculation into laboratory animals with the resultant production of the disease (Eichenwald, 1956). Although there is a generalized parasitemia present during the acute disease, isolation procedures frequently fail (Remington et al., 1962).

The methods discussed are used in the diagnosis of acquired toxoplasmosis. They may be used in the congenital form but more often conclusive diagnosis is made by the demonstration of the parasite in Wright stained smears of the newborn's spinal or ventricular fluid. Serological studies at the time of birth and 3 months later should be performed to confirm the diagnosis (Feldman, 1968b).

Newer methods in serological diagnosis and the development of the intradermal skin test have facilitated studies on the incidence of Toxoplasma in all parts of the world. Table I lists some of these studies.

Further research has been done in an attempt to correlate the incidence of Toxoplasma antibodies with geographical location (Walls and Kagan, 1967b; Walls et al., 1967a; Paul et al., 1964), occupational associations (McCulloch et al., 1963; Walls and Kagan, 1967b; Kabayashi, 1963; Murakami, 1964), urban versus rural residence (French et al., 1970; Gibson, 1956; Schnurrenberger et al., 1964), animal contact (Schnurrenberger et al., 1964; Kimball et al., 1960), and climatological variations such as altitude and rainfall (Walton et al., 1966b). Generalizations drawn from these studies are; (1) the incidence of antibodies tends to increase with age; (2) a higher incidence occurs in geographical locations with a lower elevation and a greater rainfall; (3) a higher incidence occurs in rural populations; (4) persons in contact with animals or animal products have antibodies more frequently than those who are not; (5) consumption of raw beef or eggs increases the incidence of antibodies; (6) the incidence was not influenced by sex.

Several groups (Burkinshaw et al., 1953; Cook and Derrick, 1961; Fair, 1959; Hongo et al., 1964) have published data on the occurrence

Table I. Incidence of *Toxoplasma* antibodies in various geographical locations.

Author	Date	Country	Locality	Type of group	Test used	No. tested	No. +	%.+
Feldman and Miller	1956	USA	Alaska	Eskimo	Dye test	21	0	0
"	1956	USA	Southwest	Navajo	Dye test	236	10	4
"	1956	Iceland		Normal pop.	Dye test	108	12	11
"	1956	USA	Portland, Ore	"	Dye test	293	51	17
"	1956	USA	St Louis, Mo	"	Dye test	184	47	26
"	1956	USA	New Orleans	"	Dye test	270	84	31
"	1956	USA	Pittsburgh	"	Dye test	144	51	35
"	1956	Haiti		"	Dye test	104	37	36
"	1956	Honduras		"	Dye test	266	170	64
"	1956	Tahiti		"	Dye test	121	82	68
Gibson et al.	1956	USA	Tennessee	Negro	Dye test	987	215	22
Jacobs et al.	1954	USA	New York City	Jewish	Dye test	62	9	15
Gibson	1958	Costa Rica	Turrialba	Adults	Dye test	156	138	89
Gibson	1956	USA	Memphis, Tenn	Adults	Dye test	317	75	24
Gibson	1956	USA	Memphis, Tenn	Children	Dye test	700	42	6
Gibson	1958	Guatemala	Esciunta	> 16	Dye test	100	94	94
Biagi*	Unpub	Mexico	Tampico	All ages	Intradermal	231	110	48
Jacobs et al*	Unpub	USA	Washington,DC	Negro	Dye test	124	33	27
Feldman	1965	USA	Northeast	Military recruits	Dye test	109	22	20
			North Mid-Atlantic			193	30	16
			South Atlantic			469	18	18
			East North Central			549	97	18
			East South Central			256	49	19

Table I (Continued)

Author	Date	Country	Locality	Type of group	Test used	No. tested	No. +	% +
			West North Central			315	37	12
			West South Central			201	27	13
			Mountain			182	6	3
			Pacific			406	32	8
Lamb and Feldman	1968	Brazil		Military recruits	Dye test	1455	751	52
Midtvedt	1965	Norway	All parts	Children	Dye test	320	36	11
Ludlam	1965	Africa	Niger Delt	All ages	Dye test	64	40	65
Wallace	1969	Pacific Atolls	Eauripik	All ages	Dye test	134	9	7
			Waluae	All ages	Dye test	99	43	43
			Ifalik	All ages	Dye test	105	59	56
Kobayashi et al.	1963	Japan	Tokyo	Abattoir workers	Intradermal	90	61	68
				Pluck handlers	Intradermal	137	46	34
				Ham making workers	Intradermal	84	25	30
				Normal res.	"	181	54	30
Murakama	164	Japan	Entire country	20-24 yrs	Dye test	483	35	7

* Wright, 1957

of Toxoplasma antibodies in the residents of schools for the mentally retarded. Generally they found that Toxoplasma as a cause of mental deficiency was rare. All studies, except one in Georgia, had been done outside of the United States.

Due to the fact that only one epidemiological study in a school for the mentally retarded had been done in the United States, it was advisable to undertake this type of study in Montana.

Antibody titers as determined by the Indirect Fluorescent Antibody Test and the Indirect Hemagglutination Test were correlated to the type of mental retardation, age, sex, and length of time institutionalized. Control populations were tested concurrently to determine incidence in a normal population.

MATERIALS AND METHODS

STUDY GROUPS

Group 1. 478 serums were drawn from residents selected from groups classified according to diagnosis at the Boulder River School and Hospital located at Boulder, Montana. The groups included:

(1) Chromosomal defects (XXY,XX/XO,XXY + t) and mongolism

(2) deformity syndromes, unclassified (three minor defects or more) and classified

(3) genetic, including PKU, homocysteinuria, deafness, pseudo-hypothyroidism, Mast syndrome

(4) prematurity of unknown cause and caused by attempted abortion or placenta praevia

(5) post infection, in utero, unknown, Rubella, Coxsackie B, poliomyelitis, mumps encephalitis, Western Equine encephalitis, measles encephalitis, and tubercular meningitis

(6) genetic and environmental including jaundice, kernicterus, toxemia of pregnancy and unclassified

(7) familial (no deformities) caused by consanquinity, socio-cultural deprivation, mental retardation with mental illness and aphasia

(8) trauma caused by a fall, an accident or during birth

(9) unclassified group associated with microcephaly, spasticity, epilepsy, and hydrocephalus and another group with mental retardation with no apparent cause.

Residents were selected without regard to age or sex. Blood specimens were taken and the serums were frozen.

Group 2. One portion of the control population consisted of 285 serums drawn from patients admitted to St. Peter's Hospital and Montana Crippled Children's Hospital in Helena, Montana from September 1969 through November 1969. The patients were selected randomly without consideration for sex and age, except in the children's hospital where all were under 10 years of age.

Group 3. Another portion of the control population, consisting of 197 serums was drawn from patients admitted to St. Vincent's Hospital in Billings, Montana from March 1970 through May 1970. Again, the patients were selected without regard to sex or age.

In selecting the control group from two different cities in Montana, an attempt was made to demonstrate the effect of climatological variations such as elevation and rainfall on the incidence of the disease.

MAINTENANCE OF THE ORGANISM

Toxoplasma gondii suspended in sheep red blood cells was received from the National Communicable Disease Center, Atlanta, Georgia, courtesy of Dr. Kenneth Walls. This suspension was propagated by inoculating 0.5 ml intraperitoneally (IP) into Swiss Manor mice (Walls, 1969). Thereafter, it was maintained by inoculating 0.1 ml

IP of peritoneal fluid diluted 1:100 in sterile saline into 6-8 week old animals (Eyles and Coleman, 1956a). The organism was passaged every 4 to 6 days (Fletcher, 1965), and was preserved by freezing according to the procedure of Eyles et al. (1956b). This was accomplished by collecting the peritoneal fluid from infected mice and diluting it 1:5 with a solution of 20 percent serum in physiological saline. One ml samples were placed into sterile 2 ml glass ampoules. To each ampoule, 1 ml of a 10 per cent glycerol solution was added, the contents mixed with a syringe, and the necks sealed with heat. The ampoules were frozen by placing them in a basket which had been lined with a layer of absorbant cotton approximately 1 inch in thickness. The baskets were placed in a REVCO freezer at -70°C. The suspensions were warmed in a 37°C water bath for 5 minutes and 0.5 - 1.0 ml was injected into Swiss Manor mice (Eyles et al., 1956b).

INDIRECT FLUORESCENT ANTIBODY TEST

Antigen preparation. Peritoneal fluid was collected from mice that had been inoculated 4-5 days earlier. The exudate was centrifuged at 200 rpm for 10 minutes to sediment the leucocytes. The supernatant was removed, phosphate buffered saline (PBS) pH 7.2 added, and recentrifuged at 2000 rpm for 15 minutes to sediment the organisms.

The supernatant was discarded and the organisms were resuspended in PBS pH 7.2. The wash procedure was repeated 3 times after which the organisms were suspended in a sufficient volume of PBS pH 7.2 to obtain a concentration of 25-30 organisms per high, dry, field. A small drop of the suspension was placed in each of four 1.5 cm circles inscribed on microscope slides with a waterproof, felt-tip pen. The slides were allowed to dry at room temperature and stored at -70°C (Fletcher, 1965; Sulzer and Hall, 1966).

Preparation of the anti-human conjugate. Fourteen human serums were drawn from patients randomly selected at St. Peter's Hospital in Helena, Montana. The serums were pooled prior to their inoculation into 3 randomly bred rabbits.

Three ml of serum was injected intramuscularly into each rabbit. Three similar injections followed at 10 day intervals, at the end of which the rabbits were bled from the marginal ear vein. The precipitin titers as determined by the tube test were number 1, 1:10,280; number 2, 1:5280; and number 3, 1:5280.

Each rabbit was given an additional injection of 1 ml of human serum intravenously in order to elevate the titer, after which they were: number 1, 1:20,560; number 2, 1:20,560; and number 3, 1:10,280 (Campbell et al., 1964). The rabbits were exsanguinated and the serums stored at -15°C.

An aliquot of rabbit anti-human serum was diluted with 10 volumes of distilled water to which an equal volume of saturated ammonium sulfate (Mallinkrodt) was added. The mixture was placed at 4°C overnight after which the ammonium sulfate was removed by aspiration, leaving the globulins suspended in a small volume of 50 percent saturated ammonium sulfate. A second precipitation was done by dissolving the globulins in a volume of distilled water equal to the volume of the precipitated globulins. Again, an equal volume of saturated ammonium sulfate was added to the solution. The precipitated globulins were collected by centrifugation.

For dialysis, the globulins were dissolved in a volume of water equal to the original volume of the whole serum. They were refrigerated at 4°C for 10 minutes to dissolve all the globulins. The solution was placed into dialysis tubing and dialized against 0.85 percent sodium chloride at 4°C. Two dialyses, of 12 liters each were performed, one from 1:00 p.m. to 5:00 p.m. in the afternoon and the other from 6:00 p.m. to 7:00 a.m. the next morning. At the end of the second dialysis, the 0.85% NaCl used for dialysis was checked with a 1 percent solution of barium chloride. The absence of turbidity indicated the elimination of the sulfate ion (Goldwasser and Shepherd, 1958). The globulin solution was pipetted from the tubing and the volume measured.

Carbonate-bicarbonate buffer (0.5 M pH 9.0) was added to the globulin solution in an amount equal to 10 percent by volume. The fluoroisothiocyanate (FITC) added to the globulin mixture at this point was calculated using the globulin volume after dialysis, the amount of globulin present in the dialized solution as calculated by the Biuret procedure (Bauer et al., 1968), and 20 milligrams of FITC needed per gram of protein (Fletcher, 1965; Walls, 1969).

Conjugation was performed at room temperature for 2 hours by shaking the dye-globulin mixture gently on a mechanical rotator. Unconjugated FITC was removed by filtering the conjugate through a G-50, fine, globular Sephadex column which had been calibrated with 0.02 M PBS pH 7.2. The conjugated serum was stored in 1 ml aliquots at -15°C (Fletcher, 1965).

The optimum dilution of the conjugate was determined by making dilutions of 1:20, 1:40, 1:80, and 1:100 in PBS pH 7.2. The dilutions were used in the performance of the Indirect Fluorescent Antibody Test on known positive and negative serums. The optimum dilution of conjugate was that dilution which gave maximum fluorescence at the known titer of the positive serum and negative fluorescence with the negative serum.

Indirect Fluorescent Antibody Test procedure. Initially, serums were diluted 1:16 and 1:64. Antigen slides were washed in a light stream of distilled water, blotted with laboratory tissue, and placed on wet filter paper in 15 cm petri plates. Each smear was covered with a different serum dilution, the pan was covered and placed in a 37°C incubator for 30 minutes, after which the serums were rinsed with a light stream of distilled water. The slides were immersed in a staining dish containing PBS pH 7.2 and rotated at 15 rpm for 15 minutes. The slides were blotted dry, and replaced in the petri plates. At this time, the smears were covered with the optimum dilution of anti-human conjugate which had been diluted in PBS pH 7.2. The slides were handled as above. After rotation, the slides were removed, washed with distilled water, blotted dry, and coverslipped with buffered glycerol pH 8.0 (Fletcher, 1965). The slides were examined on a fluorescent microscope (Leitz) using a BG-12 exciter and an OG-1 barrier filter (Kelen et al., 1962). With every test the following controls were used: saline, a negative serum, and a positive serum of known titer. The positive serum was tested at four dilutions, one below, one at, and two above the known titer. The negative serum was tested at 1:64 only. A reaction was considered positive when yellow green fluorescence was observed to extend around the entire porifery of the organism (Sulzer and Hall, 1966), as shown in Figure 2.