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Serological and Clinical Studies on Toxoplasmosis in Patients from Voyvodina (Yugoslavia)

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Abstract. Blood samples of about 500 individuals from 0 to 49 years of age were tested by complement fixation reaction with *Toxoplasma* antigen.

An apparent positive correlation was found between age (from the 2nd year onwards) and the per cent rate of positivity, which is reaching its peak (35 per cent) at about the 15th year of age. 17 per cent of the newborns show CF titers, mostly of maternal origin, which are eliminated during the first month of extrauterine life. About 50 per cent of seropositive mothers gave birth to seropositive infants. Possible reasons for the non-transmittance of maternal antibodies to the offspring are discussed.

Possible examples of acquired manifest toxoplasmosis, symptomless cases and congenital toxoplasmosis of infants were exemplified. The majority both of the mental retardations and of congenital heart diseases, observed in children, seem to be caused by diseases other than toxoplasmosis. However, such children tend to be infected with *Toxoplasma* at a rate significantly higher than the normal ones. Sinusitis with or without rhinitis, fever and lymphadenopathy showed a significant association with positivity of antibody test. *Toxoplasma* infection in the material observed apparently did not play a significant role as predisposing factor for pathological termination of pregnancy.

Complement-fixation (CF) tests with *Toxoplasma* antigen were conducted in Yugoslavia first in 1952. Of a total of 40 specimens tested, sera of 2 patients showed positive CF-titers, one of them being a newborn, revealing a titer of 1/32 (TERZIN at al. 1954). As shown in a bibliographical review (BABIĆ 1965), since 1952 numerous publications from Yugoslavia reported on: proved cases of human toxoplamosis as well as on successful isolations of *Toxoplasma gondii* from dogs (MARŽAN and WIKERHAUSER 1956; SIMIĆ et al. 1956), rodents (GOLOŠIN 1960) and from various species of domestic birds (SIMIĆ et al. 1961).

Systematic serological surveys of the normal population or of specific categories of sick are rare. First VALENTIČIĆ and BANIĆ (1957) and later KOZAK (personal communication) tested, both with the Sabin — Feldman (S.F.) dye test and the CF test, a great number of human sera collected in Slovenia. They found a high incidence (about 37 per cent) of positive reactors in the CF test. Also SKALICKY (1966) found a high incidence of reactors (31 to 36 per cent), both with the S. F. and the

skin test, in parturient women in Slovenia. GVOZDENOVIĆ and MILADINOVIĆ (1960) reported on results of a serological survey (S. F. dye-test) of mentally retarded blind and deaf-mute children.

The present serological survey was initiated in view of the numerous claims made about toxoplasmosis as possible cause of congenital malformations, which are known to be of high prevalence in Voyvodina. However, the first results have shifted the main emphasis on findings unrelated to the primary aim of this report. From February 12th till December 30th 1966, both from the Department of obstetrics and from the Childrens' hospital, randomly allotted patients' blood samples were sent to the laboratory to be tested for antibodies against *Toxoplasma gondii*. The results reported here are based on clinical observations made on 495 patients, from whom 504 blood samples were tested.

MATERIALS AND METHODS

Complement-fixation (CF) reaction

Blood samples were drawn from the cubital vein or from the umbilical cord. The serum specimens were preserved with sodium aziomide (final conc. 0.08 per cent) and stored at 4 °C. Sera to be tested were first inactivated at 57 °C for 30 minutes. With the *Toxoplasma* antigen we tested only sera showing no anticomplementary activity in dilutions from 1/1 to 1/4.

The *Toxoplasma* antigen was made from three-times frozen and thawed peritoneal exudate of mice, infected with RH strain of *Toxoplasma*, preserved with glycerol (final conc. 20 v/v percent). In a similar fashion was prepared also the control antigen, from peritoneal exudate harvested 6 hours after intraperitoneal injection, of a dose of typhoid-paratyphoid vaccine, containing about 250,000 killed organisms per mouse. The procedures of preparation, preservation and testing, both for specificity and sensitivity as well as for potency and stability of the antigens, will be described separately. In the diagnostic test we used two units of the *Toxoplasma* antigen per volume unit, and the control antigen was diluted in the same proportion as the *Toxoplasma* antigen. The units of the *Toxoplasma* antigen were determined by titrating it in the presence of eight antibody units of rabbit serum obtained from animals surviving intracutaneous infection with *Toxoplasma*.

With 4—8 antigen units per volume unit, and using an overnight fixation at 4 °C, the sensitivity of the test can be increased to give 8 to 16 fold higher CF-titers than those reported here. In order to keep the high specificity of the reaction we did not use overnight fixation and, for other reasons, the amount of antigen used in the diagnostic tests was 2 units per volume unit only.

Other reagents and the method of titration were those described in a preceding report (TERZIN et al. 1954). Two-fold dilutions of patients' sera, diluted in saline were titrated in dilutions from 1/2 to 1/32. On testing the same serum twice, or with two different batches of antigen, the titers obtained were mostly the same. Some sera had to be retitrated several times, and the titer obtained twice or more times subsequently was adopted as final. Nearly all sera showing a positive (1/2 or higher) titer, had been tested also with the control antigen, none of them giving a nonspecific fixation.

From 9 patients we tested also second specimens of blood. One showed a rise, another one a drop of the specific CF-titer (to be discussed later), and in 7 of the second specimens taken 6 to 9 days after the first one, the CF-titers remained unchanged.

Serum donors and clinical data

About 60 per cent both of the mothers and newborns, and about 26 per cent of the children came from the city of Novi Sad, the rest being from other parts of Voyvodina. The frequency of positive

reactors was approximately the same in the donors both from Novi Sad and from other parts of Voyvodina.

The clinical data were submitted to the laboratory before the results of the serological tests were revealed to the clinicians. This provided for less complete, but more uniform clinical examination both of the seropositive and seronegative reactors. In the age group between 0 days and 14 years, 176 (55 per cent) of the donors were males, and 143 (45 per cent) were females. The overall rate of seropositive reactors was the same in both sexes. All donors above the age of 14 years were females (mostly mothers).

None of the serum donors received blood transfusion or injection of blood-derivatives, before the serum sample to be tested had been taken.

No special stress was laid on searching for symptoms expected in cases of toxoplasmosis. The following diagnostic methods were not utilised: x-ray examination of the skull, ophthalmological search for signs of chorioretinitis, attempts to demonstrate the presence of *Toxoplasma* organisms by isolation or other techniques. Excepting the 9 patients mentioned, from all donors only single samples of serum were tested. Consequently, the data collected do not allow either for a precise differentiation between types of *Toxoplasma* infection, or for distinction of all toxoplasmosis cases from other diseases inducing similar symptoms. Consequently, the seropositive cases observed could be arranged only to one of the following 3 categories: (A) normal newborns with antibodies transmitted from mothers; (B) cases of congenital toxoplasmosis of infants; and (C) symptomless or manifest cases of acquired toxoplasmosis, or crossinfected persons displaying symptoms due to diseases other than toxoplasmosis.

RESULTS AND DISCUSSION

A. Maternal antibodies in newborns

Of a total of 164 newborns (mean age 15 days), 27 were found with CF titers ranging from $^1/_2$ to $^1/_8$ (see Table 1). Of these 27 seroreactors, 25 were apparently normal babies born in the hospital and 2 were out-patients, of poor socio-economic sta-

Table 1. Age-specific rates of seropositive reactors

Age groups (mean age)	Positives tested		Reciprocals of positive titers	
	no. of sera	percent	range	mean*
0—29 (15) d.	27/164	17	2—8	2.6
1—11 (6) m.	0/41	0	—	—
1—2 (1.5) y.	2/39	5	2	2
3—8 (5.5) y.	9/49	18	2—8	2,9
9—14 (11.5) y.	9/26	35	2—4	3,4
15—49 (32.0) y.	62/176	35	2—16	2.9
All ages	109/495	22	2—16	2.8

Legend:

* Geometrical mean.
d. = days;
m. = months;
y. = years.

tus, whose mothers refused to donate blood for examination. One of these newborns was found suspect of acute congenital toxoplasmosis.

The seropositive newborns possessed residual antibodies apparently transmitted from their mothers. The passively acquired titers seem to be eliminated from the infant during the first month of its extrauterine life. This claim is supported by the fact that the difference between the two proportions observed in the first and second age-group (27/164 and 0/41 respecively), proved to be statistically significant (see Table 1).

Table 2 shows two-way frequencies of the reciprocals of CF-titers found in 153 pairs (306 samples) of mother-newborn serum specimens. Ninety five pairs (190 specimens) of mother-newborn sera gave negative results (CF-titers $< 1/2$). Of the remaining 58 pairs, both mother and newborn were positive in 23 pairs, the mother only in 33 pairs, and the newborn only in 2 pairs.

In 14 pairs of sera the CF-titers were the same both in mother and newborn. In 41 pairs the CF-titer found in the mother was higher than in the newborn. Of these, 28 of the mother sera showed 2-times, 10 sera 4-times, and 3 sera 8-times higher CF-titer than that of their newborn (titer of negative specimens taken as 1/1). In 3 pairs only, the CF-titer was higher in the newborn than in its mother. The CF-titers found in these 3 newborn serum specimens were only two-times higher than the CF titers revealed by their mothers. The proportion 3/44 when tested by chi-square test, proved to be significantly different ($p < 0.001$) if compared with the proportion 22/44, taken as zero hypothesis (see Table 2). These findings indicate,

Table 2. Reciprocals of CF-titers found in paired serum samples of mothers and newborns

Mothers	Newborns				
	<2	2	4	8	Total
<2	95	2	—	—	97
2	21	10	1	—	32
4	9	6	4	—	19
8	3	1	—	—	4
16	—	—	—	1	1
Total	128	19	5	1	153

that the transmittance of mother antibodies to the newborn is significantly limited. Also the data presented in Table 1 support this claim. Proportion 27/164 observed in the first age group proved to be significantly different from proportion 62/176 observed in the last age group, comprising all mothers (chi-square value $= 14$, $p < 0.001$).

These findings give rise to the question, why one out of every two sero-positive mothers does not transmit antibodies to the offspring. At least three categories of cases seem to be responsible for that (a, b, and c).

(a) In cases of past infections, the low CF-titers of maternal 7 S antibodies could be transferred to the newborn in amounts not detected by our CF-test of low grade sensitivity. (b and c) Cases when in the mothers are circulating 19 S antibodies which are known not to be transmitted by the foetal membranes. The 19 S globulins could be produced during pregnancy either (b) as primary response to acute *Toxoplasma* infection of the mother (DESMONTS, COUVREUR and BEN RACHID 1965), or (c) they could reappear during pregnancy of chronically infected women (REMINGTON and MILLER 1966).

B. Possible cases of congenital toxoplasmosis

Of the 244 infants belonging to the first 3 age groups, shown in Table 1 (infants below the age of 3 years), only two were suspect of congenital toxoplasmosis. As yet, neither samples of their mothers' blood nor second spacimens of these two infant's blood could be procured for testing. The findings referring to cases 1 and 2 presented in Table 3 seem to be favouring a diagnosis of congenital toxoplasmosis.

It is known that acute congenital infection with *Toxoplasma* takes place only in mothers with acute infection, estimated to occur in less than 1 per cent of all acquired infections (DESMONTS, COUVREUR and BEN RACHID 1965). Even of these cases a proportion of congenital *Toxoplasma* infections may take a clinically mild or inapparent course (HEDENSTROEM 1957; DESMONTS, COUVREUR and BEN RACHID 1965). Consequently, in our group of only 41 observed infants of a mean age of 6 months (second age group in Table 1) positive reactors exemplifying cases of congenital toxoplasmosis should not be expected.

C. Examples of acquired toxoplasmosis

Table 3 presents the clinical findings referring to 20 CF-positive children of the age from 2 to 15 years (cases no. 3 to 22). Some of the 20 seropositive chidren are possibly manifest (e.g. cases l0, 12, 14 and 15) or symptomless (e.g. cases 18 and 21) cases of acquired toxoplasmosis, others are children crossinfected with toxoplasmosis, presently displaying symptoms possibly due to diseases other than toxoplasmosis (e.g. cases 6, 11 and 19). None of the 62 seropositives in the age group from 15 to 49 years (see Table 1) except one (case 22 listed in Table 3), showed clinical symptoms suggesting recent or past infection with *Toxoplasma*.

D. Association between clinical manifestation and seropositivity

Although insufficient for diagnosing all individual cases of the seropositive reactors, the available data allow for useful conclusions based on an analysis of grouped data.

In Table 4 we compared the frequency rates of several clinical diagnoses observed both in the CF-positive and CF-negative group of children (altogether 164

Table 3. Clinical findings in newborns and children

Patients	Age y.	Age m.	CF-titer 1/X	Mental retardation	Sinusitis	Fever	Lymphadeno-pathy	Pneumonia	Splenohepato-megaly	Other findings
1	—	1	4	—	—	++	—	—	—	p. premat; icterus
2	1	10	2	+	—	+	+	—	+	thrombocytopenic purpura;
3	2	—	2	—	—	++	—	+	+	rhin;
4	3	2	2	—	—	—	+	—	—	haemophilia A;
5	3	3	2	+	—	++	—	—	+	bronchopharingitis;
6	3	4	2	—	—	+	—	—	—	rhinopharingitis;
7	3	8	4	+	+	++	—	—	—	men-enc; rhin
8	5	3	4	—	+	—	+	+	—	
9	5	5	4	—	+	++	—	+	+	
10	5	10	2	—	+	++	+	+	+	
11	6	3	2	—	—	—	+	—	—	canker sores;
12	7	1	8	—	—	+	—	+	+	after 33 d. CF 1/1; myocarditis
13	9	2	2	+	—	+	+	+	—	chd.;
14	10	—	4	—	+	—	+	—	—	impaired vision;
15	10	1	4	+	—	++	+	—	+	after 8 d. CF 1/8
16	10	5	4	—	—	—	—	—	+	
17	10	9	4	—	+	++	—	+	—	
18	10	10	4	—	—	—	—	—	—	pharingitis;
19	11	4	2	—	—	++	—	—	—	polyarthritis;
20	12	—	4	—	+	—	+	—	—	cystitis;
21	12	2	4	+	—	—	—	—	—	chd.;
22	15	—	2	+	+	—	—	—	—	chd.; rhin; mongolism;

Legend:

y. = years; m. = months;
p. premat = partus prematurus;
rhin = rhinitis;
men-enc = meningo-encephalitis;
d. = day;
chd. = congenital heart disease.

children). The age of the observed children varied from 0 month to 15 years, 95 per cent of them being between 1 and 14 years. Data in Table 4 indicate a significant association between mental retardation and crossinfection with toxoplasmosis. They do not answer, however, if the mental retardations were caused by the infection with *Toxoplasma gondii*, or if these infections took place at a higher rate in mentally retarded persons, due to neglect of personal hygiene or other factors responsible for a higher infection rate in mentally retarded persons. Some of the children listed in Table 3 (cases 13, 15, 21 and 22) developed *Toxoplasma* antibodies possibly after having acquired mental retardation and congenital heart disease due to causes other than toxoplasmosis.

On significantly larger groups of children than ours, LABZOFFSKY and his associates (1965) found no difference between the frequency of CF-positive reactors in normal and mentally retarded donors. Assuming that our retarded patients lived under less cotrolled sanitary conditions than those in Canada, it appears to us plausible to conclude that mentally retarded children, under certain conditons, can be infected with toxoplasmosis at a higher rate than normal persons of the same age. Data from Table 4 show that neither our cases of congenital heart disease, nor those of hydro- and/or microcephaly showed any association with crossinfections with toxoplasmosis.

Table 4. Number (per cent) of children with various clinical manifestations

Compared groups of seroreactors	All cases	Mental retardation	Sinusitis a/o rhinitis	Sinusitis	Fever	Lymphadeno-pathy	Pneumonia	Meningoen-cephal.	Spleno a/o Hepato-megaly	Congen. heart disease	Hydro a/o Microcephaly
CF positives	22 (100)	7 (32)	10 (46)	8 (36)	13 (59)	9 (41)	7 (32)	1 (5)	8 (36)	3 (14)	0 (0)
CF negatives	142 (100)	13 (9)	27 (19)	22 (15)	51 (36)	33 (23)	29 (20)	2 (1)	50 (35)	6 (4)	4 (3)
Significance* of difference between pairs of frequencies		<0.01 >0.005	<0.01 >0.005	<0.05 >0.02	<0.05 >0.02	<0.1 >0.05	n.s.	n.s.	n.s.	n.s.	n.s.

Legend:

a/o = and / or.
* Expressed as probability range (p) for the chi-square value obtained.
n.s. = not significant.

Of the most common symptoms, observed in our group of seropositive patients, the following showed significant association with positive serological response:

sinusitis and/or rhinitis, sinusitis (without rhinitis), fever and lymphadenopathy (observed at a frequency at the borderline of significance).

The latter two symptoms are known to be associated with cases of acquired toxoplasmosis, and a possible association of sinusitides (with or without rhinitis) with cases of acquired toxoplasmosis seems to us worth while for further study.

Although claims about possible transmission of toxoplasmosis by droplet infection are mainly conjectural, they can not be excluded. Of the respiratory symptoms observed in cases of acquired toxoplasmosis, many authors mention cough due to pneumonia, and some rhinitis as well (e.g. HEDENSTROEM, HULDT and LAGERCRANTZ 1961). The reactivity of the paranasal sinuses to agents invading the nasopharyngeal space, is a well known clinical observation. Collection of lymphatic tissue beneath the epithelium is a characteristic feature of the nasal sinuses. GARD and MAGNUSSON (1951) and SIIM (1961) pointed out the important role played by the lymphatic tissue both in the clinical manifestation and in pathogenesis of acquired toxoplasmosis in men. All these circumstantial evidences seem to support the validity of our observation on the association of sinusitis (with or without detectable signs of rhinitis) with cases of acquired toxoplasmosis.

Table 5. Termination of pregnancies observed

Present partus	Abortion in the past	Number (per cent) of cases	
		CF negatives	CF positives
maturus	—	92 (85)	56 (93)
prematurus	—	12 (11)	3 (5)
immaturus	—	3 (3)	1 (2)
—	recorded	22 (21)	10 (17)
—	not recorded	85 (79)	50 (83)
All cases		107 (100)	60 (100)

As shown in Table 5, the termination of the observed pregnancies as partus maturus, prematurus or immaturus took place at comparable rates, both in the group of CF-negative and CF-positive patients. Also the proportion of cases with a history of one or more abortions in the past was comparable in the two groups of seroreactors. The difference observed between pairs of the compared frequencies was in no case significant ($p > 0.2$). These findings seem to indicate that, in our material, infection with toxoplasmosis did not play a significant role as a predisposing factor for pathological termination of pregnancy.

E. Prevalence of crossinfections with toxoplasmosis

Data in Table 1 show that after the lst month of life the frequency of seropositive reactors increases with growing age, the uppermost percentage rate (35 per cent) being reached at the age of about 14 years.

The coefficient of correlation, between the mean age in years and the per cent rate of seropositive reactors, found for groups with mean age of 0.5, 1.5, 5.5 and 11.5 years, amounts to 0.996 (0.01 p 0.001), indicating a positive correlation between age and frequency of seropositive reactors.

The frequency of reactors to *Toxoplasma* antigen was studied mostly with the S.F.-dye test and the skin test. Blood samples collected from normal population, in various parts of the world, showed great divergence in the prevalence of positive S. F.-dye test reactors, varying from zero to nearly ninety per cent (FELDMAN and MILLER 1956; DESMONTS, COUVREUR and BEN RACHID 1965; CARTER and FLECK 1966, and others). FUCHS, JÍRA, BOZDĚCH and JÍROVEC (1962) found 26 per cent positives of the 425 puerperal women, tested by complement fixation test. The prevalence of CF-positives we found in Voyvodina (35 per cent) for the age group from 15 to 49 years, seems to be well comparable with that reported by VALENTINČIČ, BANIČ and KOZAK in Slovenia (37 per cent). A prevalence as high as this seems to stress both the possible significance of toxoplasmosis for the public health and the need for its more extensive study in Voyvodina.

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