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The Fluorescent Staining Complement for the Serodiagnosis of Toxoplasmosis

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Abstract. The procedure of the fluorescent staining complement (FSC) adapted for use in the serodiagnosis of toxoplasmosis is described. Good results can be achieved only with guinea-pig sera and antiguinea-pig conjugate, which are free from toxoplasmic antibodies. The conjugate can be cleared of these antibodies by saturating it with toxoplasmic antigen. In our experiments we found the FSC as sensitive as the indirect fluorescent antibody test and 6.6 times more sensitive than the CFT. The FSC can be used also in anticomplementary sera.

The fluorescent antibody staining technique by COONS has been successfully applied by various authors for the serodiagnosis of toxoplasmosis. Contrary to GOLDMAN (1957a, b, 1962) who proved the presence of toxoplasmic antibody by the fluorescent inhibition test (FIT), most of the others are in favour of the indirect fluorescent antibody test (IFAT) (KRAMÁŘ 1961, KELEN et al. 1962, DESARAM et al. 1962, MANDRAS et al. 1962, KRAMÁŘ 1963, GARIN et AMBROISE—THOMAS 1963, KRAMÁŘ et al. 1964, CAMARGO 1964, FULTON et VOLLER 1964, STADTSBAEDER et al. 1964, FLETCHER 1965, SUZUKI et al. 1965, VAN NUNEN et VAN DER VEEN 1965). In sensitivity IFAT comes next to the SABIN—FELDMAN reaction, some authors consider it even of the same sensitivity (GARIN et AMBROISE—THOMAS, CAMARGO, FLETCHER, SUZUKI et al.). The only disadvantage of the IFAT is the specific conjugate needed for each species of the sera examined, hence its lesser suitability for epidemiological examinations, where toxoplasma antibodies have to be determined in the sera of different animal species. For such instances, the method proved to be the best in our hands is the FSC, introduced to the immunofluorescent techniques by GOLDWASSER et SHEPARD (1958). The principles of this method are illustrated on Fig. 1.

The method by GOLDWASSER et SHEPARD is based on the observations by LIU et HEYL (1957), who found during the study of the virus of a primary atypical pneumonia that in the IFAT, the fluorescence of the antigen increases when fresh normal human or guinea-pig sera are added to the examined serum. They assumed

a co-ordination of a complement in this reaction and confirmed the correctness of their assumption in experiments with *Rickettsia mooseri*. Using an antiguinea-pig conjugate they proved by the IFAT that even the guinea-pig complement becomes fixed to the complex of antigen plus antibody. In addition they also proved the fixation of the complement in some work with other viruses. The fluorescent staining of the complement proved most convenient not only for the fact that only a single antiguinea-pig conjugate is required for its performance, but also that the titers were approximately 4-times higher than those of the CFT. Therefore, GOLDWASSER et KISSLING (1958) proved by this method the Rabies virus, KLEIN et BURKHOLDER (1959a, b) employed this method in their studies of an experimental anaphylaxis of the rat's kidney. For proving the true fixed complement they employed conjugated antibody against the complement fixed to the stromata of the erythrocytes of rams. They called the complement, characteristic for a high specificity in the proof of the fixed complement, the anticomplement, avoiding thus the danger hidden in the employment of the guinea-pig sera, i.e. that, apart from the complement, this serum may contain even antibody against the tested antigen. In such case the antiguinea-pig conjugate reveals specific antibody against the given antigen, but not the fixed complement. Also the paper by KLEIN et COLLI (1961) deals with the preparation of the anticomplement. MÜLLER (1960, 1961, 1962a, b) employed the conjugated anticomplement in a number of works concerned with the diagnosis of the virus.

Fig. 1. Schematic representation of complement staining with fluorescent antibody. A — antigen, AN_A — anti-A-antibody, C — complement, AN_c — anti-C-antibody, F — labelled fluorochrom.

MIKHAILOV et DASHKEVICH (1961) proved microbial antibody by the FSC. Leptospirae were demonstrated in experimentally infected chick embryos by the FSC by MAESTRONE et COFFIN (1961) and by COFFIN et MAESTRONE (1962). GILLISEN (1963) stained mycobacteria by this method.

In the majority of works, the FSC was employed for proving the antigen and only in some few instances it was used for the serodiagnosis. Apart from the mentioned papers by GOLDWASSER et SHEPARD and by MÜLLER, a very interesting study on this subject was published by JENTSCH (1964), who described the use of the FSC for the determination of *Brucella* antibody in bovine sera.

Up to date, the FSC has not been employed for the serodiagnosis of toxoplasmosis. Only KELEN et al. (1962) tried to use it but found it not satisfactory because of the low intensity of fluorescence of the antigen and because of its questionable specificity. As the CFT is commonly used in the serodiagnosis of toxoplasmosis, we considered it necessary to re-examine the data by KELEN et al. Our results indicated that the FSC can be used for proving toxoplasma antibody in the sera. The methods used for the performace of this reaction and its relation to the CFT are discussed in the present paper.

MATERIAL AND METHOD

Sera: Mostly human sera were examined. Those were obtained by courtesy of Dr. J. JÍRA from the toxoplasma laboratory of the Institute of Parasitology, Czechoslovak Academy of Sciences, Prague. In addition, some sera of rats, rabbits and dogs were also examined by the FSC. All sera were kept at a temperature of —20 °C and inactivated before the performance of the FSC. Antigen was prepared after GOLDMAN (1957) as described in earlier papers by KRAMÁŘ (1963), KRAMÁŘ et al. (1964) and kept at a temperature of —20 °C.

Conjugates: we used the commercially produced FITS conjugates (Institute of Sera and Vaccines, Prague) i.e. the goat-antiguinea-pig conjugate and the rabbit-antiguinea-pig-conjugate, for the IFAT the goat-antihuman conjugate. The conjugates were saturated with liver powder to avoid any unspecific fluorescence and re-examined by the IFAT with a toxoplasma antigen to confirm the absence of toxoplasma antibody. If antibodies were found we removed them by saturation with the toxoplasma antigen, prepared in a similar way as the antigen designed for transfer to the microscope slides. The antigen (toxoplasma from the peritoneal exudate of mice fixed in 1 % formalin) was washed in phosphate buffered saline (PBS) (phosph. 0.01 M, pH 7.2). After centrifugation, 2 ml of the conjugate, well mixed with the toxoplasmas, was added to about 0.2 ml of sediment. The suspension, rocked occasionally, was left for 60 min at room temperature and then kept overnight in the refrigerator at a temperature of 4 °C. In the morning, the toxoplasmas were separated by centrifugation, the conjugate re-examined by IFAT to detect toxoplasma antibodies, which would have to be removed by more saturation. We usually found one saturation quite satisfactory.

Complement (C): the guinea-pig serum has also to be completely free of antitoxoplasma antibody; mostly, such serum is very difficult to obtain in view of the high percentage of guinea-pigs from the laboratory breeds being infected with avirulent strains of toxoplasma. This problem will be discussed later. Therefore, the presence of toxoplasma antibody had to be confirmed by the IFAT on small samples of guinea-pig blood (approx. 0.5 ml) or on dry blood on filter paper, obtained from an incision in the animal's ear (KRAMÁŘ 1964). Separately kept negative guinea-pigs are the source of complement for a long time. Young guinea-pigs aged 3—4 months were found more suitable for our purpose because of being less infected. The blood from these negative guinea-pigs was obtained by heart puncture, the sera were stored at a temperature of 4 °C. The utmost limit for using these sera was a fortnight. We also tried to conserve guinea-pig sera, but so little is needed that the use of fresh sera seems more suitable.

The performance of the serological reactions: a) the CFT was performed in the toxoplasma laboratory of Dr. JÍRA by a method described in papers by JÍRA et BOZDĚCH (1960) and BOZDĚCH et JÍRA (1961). b) IFAT: the sera at the required dilution were dropped on the antigen and incubated in the moist chamber for 30 min. After removing the surplus serum, the preparation was washed in PBS on the vibration shaker for 10 min, under tap water for 5 min and was then left to dry at room temperature. The antigen was covered with a mixture of antihuman conjugate and Evans' blue (the most suitable mixture was: 9 parts of commercial conjugate diluted 1 : 10 and one part of 0.1 % solution of Evans' blue in PBS) and incubated for 30 min at 37 °C. Then followed a washing in the same way as after the action of the sera and finally the mounting in buffered glycerol, pH 7.5. c) FSC: the guinea-pig complement was diluted with a saline solution cooled down to 4 °C (veronal, pH 7.4) of the following composition: NaCl 85 g, acidum diaethyl-barbituricum 5.75 g, natrium diaethyl-barbituricum 3.75 g, M $MgCl_2$ 5.0 ml, M $CaCl_2$ 1.5 ml, all dissolved in 1,500 ml of 60 °C hot distilled water, then cooled and more distilled water added up to 2,000 ml. Before use, the solution was diluted with distilled water to 1 : 4. Also the examined sera were titrated with this solution, starting dilution 1 : 10. 0.1 ml of the diluted complement was then mixed with 0.1 ml of the diluted serum, doubling the final solution of the serum. The antigen was covered with a well stirred mixture of serum and complement and incubated in the moist chamber for 30 min at 37 °C. Then followed the washing as described for the IFAT and the drying at room temperature. Next, we applied the titrated antiguinea-pig conjugate (the commercial conjugate was usually dil-

uted by 1 : 10), mixing it with a solution of Evans' blue (mostly 1 : 5,000) in PBS (phosph. 0.001 M pH 7.2). The antigen was covered with this mixture and incubated for another 30 min in the moist chamber at 37 °C. Then followed the washing in the same way as after the action of the sera. When dry, the preparation was mounted in buffered glycerol.

Controls: a) the following controls had to be performed for the FSC: 1) reaction with the known positive serum; 2) reaction with the proved negative serum; 3) reaction with the solitary complement diluted by 1 : 20 (when positive, it contains the complement of toxoplasma antibody); 4) reaction without the serum and without the complement, using only the conjugate plus Evans' blue (when positive, containing conjugate of toxoplasma antibody). b) Controls of the IFAT: 1) reaction with the known positive serum; 2) reaction with the proved negative serum; 3) reaction without the serum, only with the conjugate and Evans' blue. These controls should be always made before examining any new suspect sera.

Microscope: The optical system used for reading the fluorescent reactions was the Soviet microscope ML-2 with an exciter filter FS-1 (2 mm) in combination with the filters BS-8-2 and SZS-7-2 and the barrier filter ŽS-18-2; the ultraviolet radiation was supplied by the super pressure mercury lamp DRŠ-250. The intensity of fluorescence was evaluated after the following scale:

0 = toxoplasma with red fluorescence on the whole surface
± = toxoplasma with red fluorescence on the whole surface, but with signs of a weak peripheral green fluorescence at one pole only
\+ = toxoplasma with distinctly green, but weak peripheral fluorescence and with red fluorescence inside
\+ + = strongly green peripheral fluorescence, inside red
\+ + + = bright green peripheral fluorescence, green surface of toxoplasma, at some places covering the red fluorescence of the cytoplasma.

Determination of antibody titers: the dilution of sera on the antigen showing a fluorescence intensity marked + was considered the titer reacting to both the FSC and the IFAT. The sera were diluted in a twofold geometric progression starting from 1 : 10; the antibodies were determined up to their highest dilution.

RESULTS

The examinations of 70 human sera by the FSC, IFAT and CFT are reviewed in Tab. 1. At first sight the table shows the higher sensitivity of both fluorescent reactions when compared with the CFT. This fact is illustrated on the negative reactions of the sera (CFT—44 %, FSC—only 14 %, IFAT—only 11 %). The differences between the FSC and IFAT reactions are very slight, concerning generally one or two dilutions, most exceptionally three to four dilutions. For comparison see Tab. 2.

Table 2 shows that the titers revealed by the FSC are in slightly more cases higher by 1—2 dilutions; contrary to that the FSC is 6.6 times more sensitive than the CFT and the IFAT 6.7 times more sensitive than the CFT when comparing the geometric mean reciprocal titers of all three reactions. Practically, in comparison with the sensitivity of the CFT, the sensitivity of both fluorescent reactions is the same.

We also examined four human sera, which had been anticomplementary with the CFT, with immunofluorescent reactions and received the following results:

Nr. of serum	Titers with	
	FSC	IFAT
318/66	1 : 20	1 : 10
298/66	1 : 160	1 : 80
167/66	neg.	neg.
168/66	1 : 10	1 : 10

Table 1. Survey of results of the CFT, FSC and IFAT, serving as a basis for the calculation of the GMRT

Titer	CFT	Number of sera in	
		FSC	IFAT
Neg.	31	10	8
1 : 10	9	–	1
1 : 20	9	6	11
1 : 40	6	17	8
1 : 80	7	8	17
1 : 160	3	15	15
1 : 320	3	7	3
1 : 640	1	5	4
1 : 1,280	–	2	2
1 : 2,560	1	–	1
Total:	70	70	70
GMRT*)	8.206	53.71	55.14
$\frac{\text{GMRT FSC}}{\text{GMRT CFT}} = 6.6$		$\frac{\text{GMRT IFAT}}{\text{GMRT CFT}} = 6.7$	

*) GMRT – geometric mean reciprocal titers calculated after the formula

$$\text{GMRT} = \text{antilog} \frac{\Sigma f \log x}{n},$$

x — the given titer of the serum
f — number of sera of the given titer
n — number of all sera (DIGGS et SADUN 1965 after WAUGH 1952).

Apart from this we tried the FSC on a smaller number of sera of some animals, where neither the results of the CFT nor the results of the IFAT were avaible because of a shortage of the appropriate conjugates. For an orientation we are

recording the titers of toxoplasma antibody obtained with the FSC: Rats immunized with toxoplasma: 640, 1,280, 160. Dogs (unimmunized): 160, 80, 20. Rabbits (unimmunized): 640, 160, 320, 40.

Table 2. Comparison of the sensitivity of FSC and IFAT on 70 human sera

The same	FSC compared with IFAT	
	higher by	lower by
30×	1 dilution −21× 2 dilution − 4×	1 dilution −8× 2 dilution −5× 3 dilution −1× 4 dilution −1×

DISCUSSION

Our results show that the FSC is a very suitable reaction for serodiagnostic purposes and the results are the same as with the IFAT. In one point, however, it is more advantageous than the IFAT, because only a single antiguinea-pig conjugate is needed for its performance with the serum of any animal species. As shown in positive reactions performed even with sera anticomplementary with the CFT, the FSC is suitable for determining toxoplasma antibody in even these sera. This fact may be attributed to the surplus of complement in the reaction mixture with such sera, which the anticomplementary serum could not destroy.

According to our experiences, the disadvantage of this reaction rests with the fact that some conjugates, principally the guinea-pig sera, serving as a source of complement, contain frequently toxoplasma antibodies, resulting from the high infection rate of toxoplasma in these animals. This may be considered one of the main reasons why KELEN et al. (1962) did not find the FSC suitable for their work. A saturation of the antiguinea-pig conjugates with the toxoplasma antigen eliminates these unspecific reactions with relative ease. More difficult is the situation with the guinea-pig sera, because the IFAT revealed in a majority of guinea-pigs from the laboratory breeds toxoplasma antibody in relatively high titers. Thus, in a batch of 22 guinea-pigs only six were negative; in the remaining 16 animals IFAT demonstrated toxoplasma antibodies in titers ranging from 1 : 20 to 1 : 320. In another batch of 18 guinea-pigs only one was negative. Here, the saturation with a toxoplasma antigen would be useless because of a simultaneous saturation or substantial decrease of the content of complement. However, the number of negative animals needed is so small because of the little complement required for the performance of the FSC, that a few of them are quite sufficient. The rest can be used for obtaining the complement for the CFT to determine other antibodies. Apart from these facts there is the advantage of examining by the IFAT sera of guinea-pigs used for the CFT in the serological diagnosis of toxoplasmosis.

Most suitable for suppressing unspecific staining in the FCS has been found Evans' blue when mixed with the titrated conjugate. It shortens the time needed for the elaboration of the sera and the results, according to our experiences, are more definite than those obtained by using counterstain after the treatment with the conjugate. Instead of Evans' blue, trypan blue or methylene blue can also be used.

On the grounds of the experimental data presented, the FSC can be recommended for the serodiagnosis of toxoplasmosis as a further suitable reaction, which is as sensitive as the IFAT and more sensitive than the CFT.

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