FOLIA PARASITOLOGICA (PRAHA) 28: 131–136, 1981.

APPLICATION OF THE INDIA INK IMMUNOREACTION FOR THE DIAGNOSIS OF TOXOPLASMOSIS

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Abstract. The immunoreaction using India ink is proposed for the diagnosis of toxoplasmosis. The simplicity of the test and the modest equipement needed make it especially valuable for a large-scale screening.

Geck (1971) and Geck and Novák (1972) introduced a novel method for the investigation and antigenic detection of bacteria and yeast based on the binding of India ink particles to an antigen in the presence of specific antibodies. Waller (1977) followed by Kellett and Bywater (1978) used the same principle in the "India ink immunoreaction" (IIR) for diagnosing rabbit encephalitozoonosis.

Our aim was to develop the same principle for the diagnosis of human toxoplasmosis. Although our experience with this reaction was not very satisfactory in the beginning, further research proved that a modification of the IIR is a useful test for the detection of antibody to *Toxoplasma*. The results obtained by the IIR were compared with those obtained by the indirect fluorescent antibody technique (IFAT).

MATERIAL AND METHODS

Antigen. The zoites of a virulent strain R of *Toxoplasma gondii* (local origin, maintained in Institute of Parasitology, Czechoslovak Academy of Sciences) were obtained from the peritoneal exudate of laboratory mice 3 days p.i. The zoites were preserved by treatment with neutral 1 per cent formalin for 90 minutes, washed thoroughly with physiological saline and applied in drops to slides (8 drops per slide). The drops were dried and slides were stored in a freezer at −20°C. According to our experience slides prepared in this way can be used for at least 6 months (longest period tested). The same antigen was used for both the IIR and the IFAT.

Test sera. The examined sera were obtained either from individuals with suspected toxoplasmosis sent to our department for investigation or from various hospital laboratoires (biochemistry, children's hospital, blood transfusion unit, etc). Only some sera were inactivated, but, as it will be discussed later, the inactivaton had no effect on the results of IIR or IFAT.

IIR reaction. All steps of the reaction were performed at room temperature. The diluted serum was applied dropwise to the slide with the dry antigen. After 30 minutes the serum was washed away in three succeeding steps: 1) by a gentle stream of tap water, 2) by continuous agitation in phosphate buffered saline (pH 7.2) for 10 minutes and 3) by rinsing in distilled water. Then the slide was allowed to dry. Next, the complement (dilution 1 : 10, but dilutions of 1 : 10 or 1 : 100 were equally effective) was applied to the antigen for 15 minutes, another washing was performed as before, and the slide was again allowed to dry. Undiluted India ink was then applied to the preparation. After 5 minutes the India ink was washed away by a vigorous stream of tap water. The final rinsing was performed in distilled water and the slide was then allowed to dry. The result of the reaction was read under an oil immersion objective without cover-glass. For better orientation it is convenient to mark the edges of the antigen drops with a marker line made on the bottom of the slide before observation.

Complement. Lyophilized guinea pig complement (Alexin, SEVAC Prague) free of *Toxoplasma* antibody. *Toxoplasma* complement for CFT (SEVAC Prague) was equally suitable.

India ink. Selected batches of Higgins Black Magic Ink (A. W. Faber-Castell, Stein bei Nürnberg, GFR) were used.

IFAT technique. The IFAT reaction was performed in a standard way in which diluted serum was allowed to react with antigen for 30 minutes at 37 °C. The diluted (1 : 4—1 : 8) conjugate SwaHu (Swine anti Human/FITC, SEVAC Prague) was applied under the same conditions and the slides were washed as in the IIR. Finally, the slides were counterstained with Evans Blue (1 : 10 000) for 2 minutes, rinsed in distilled water, dried and read under an oil immersion objective. A fluorescent microscope ML-2 of Soviet origin, provided with high pressure mercury lamp DRSH 250 with filters BS 8-2, FS 1-2, S3S 7-2 was used.

RESULTS

When the IIR is performed with sera containing specific antibodies, the *Toxoplasma* zoites are "stained" very dark with the India ink and are clearly visible against the faint background staining which always covers the area to which the India ink was applied. The staining is, of course, more conspicuous at the edges of the organisms.

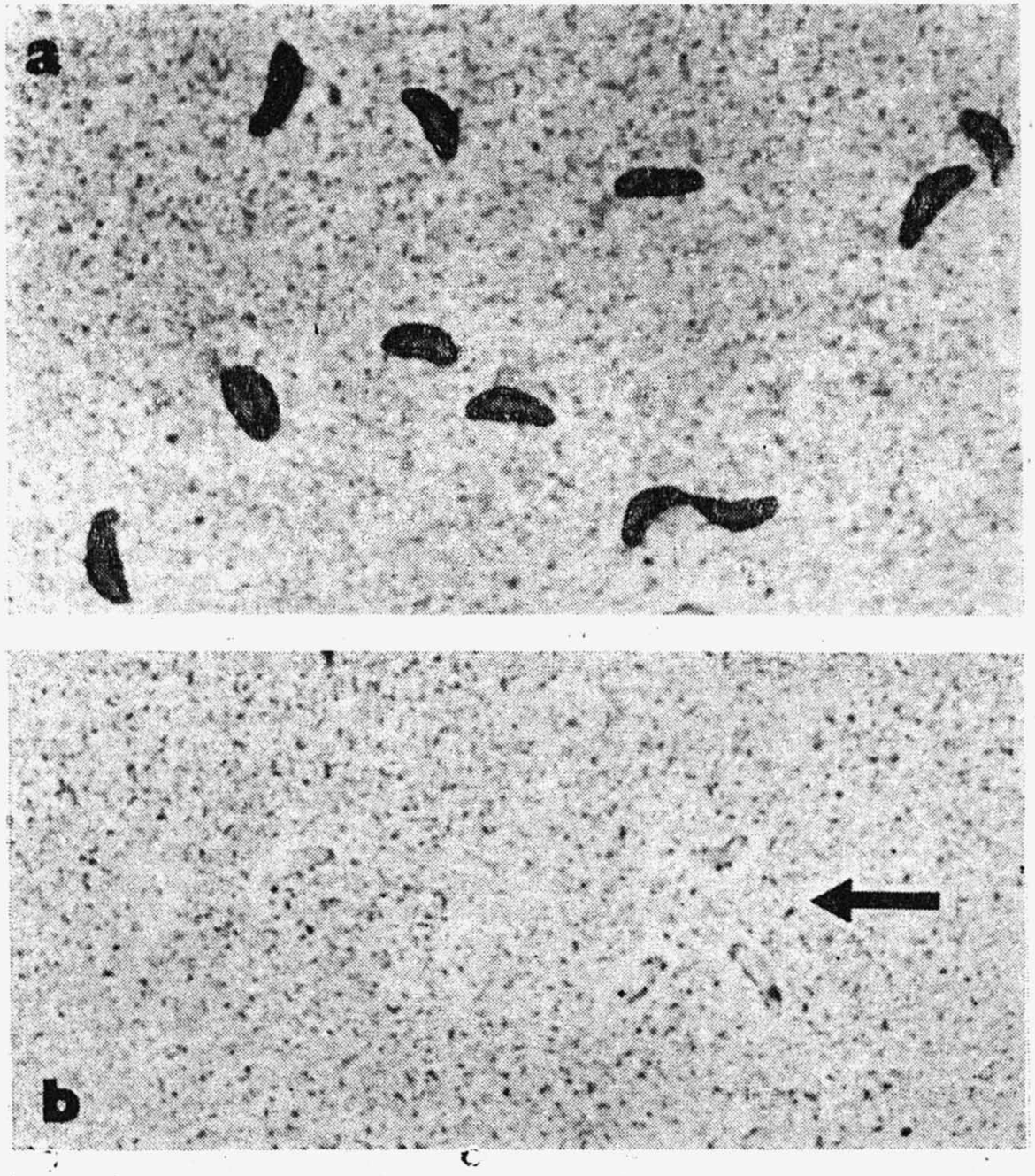

Fig. 1. *Toxoplasma gondii* zoites as seen in different results of India ink immunoreaction. (a) — Positive and (b) — negative reaction. On (b), one group of zoites marked by an arrow.

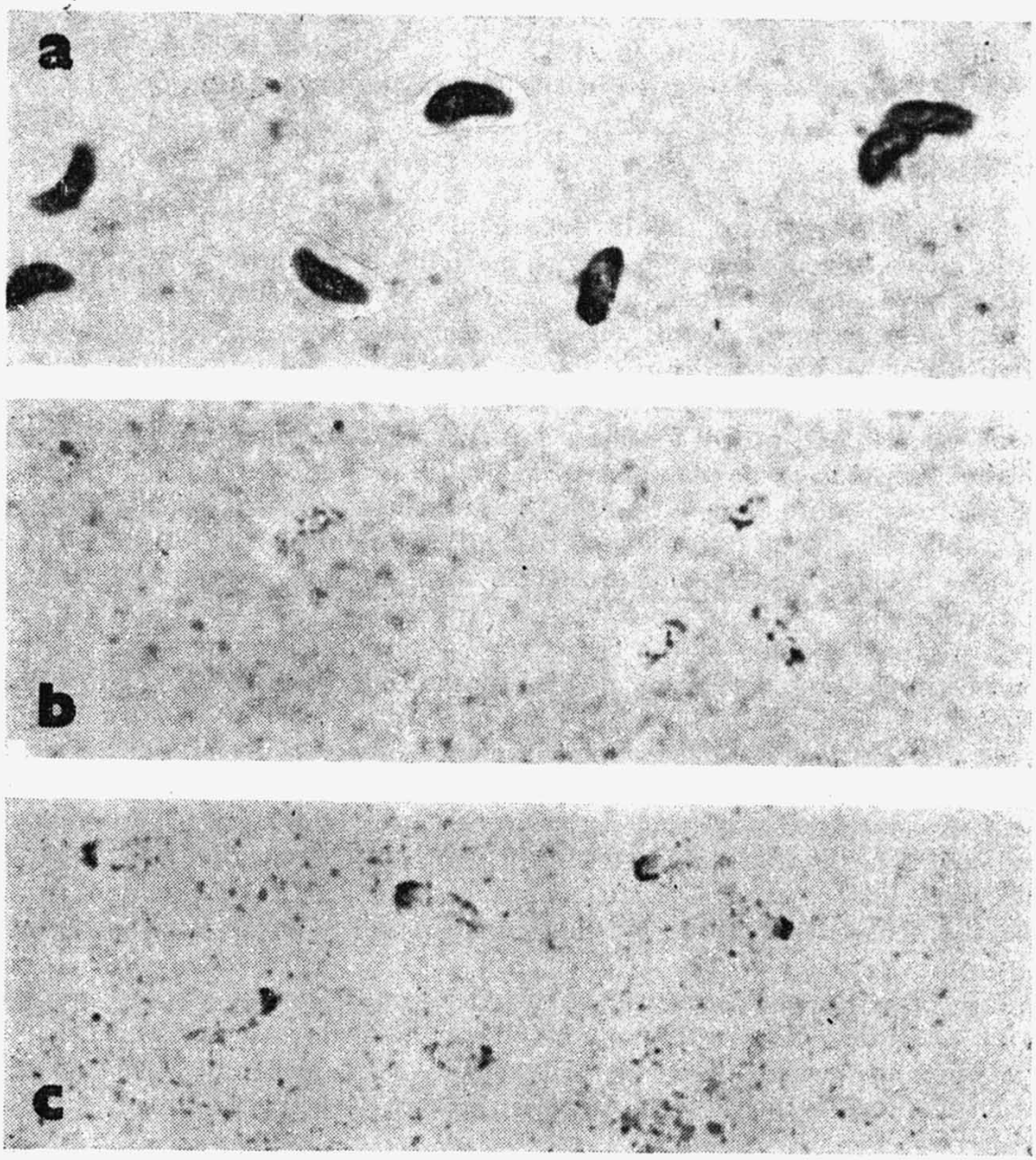

Fig. 2. (a, b) — The same as in Fig. 1, but focused on the upper surface of *Toxoplasma* zoites. The surface binding of India ink is observable. (c) — "Polar staining" or "cap phenomenon" in the IIR.

A semiquantitative evaluation of the reaction has been made according to the following arbitrary scale:

+++: the zoites are heavily stained with conspicuously dark outlines (Fig. 1a)
++: the zoites are less conspicuously stained
+: only the outlines of zoites are stained
0: the zoites are nearly invisible against the background or sometimes the body outline is slightly, irregularly stained (Fig. 1b).

The "cap phenomenon" (or "polar staining)" known from the IFAT is also observable in the IIR (Fig. 2c).

We have examined a total of 533 sera using both the IIR and the IFAT. Of them 253 sera were examined in full range of dilutions (1: 2 — 1: 4096, except 1 : 4), while 280 sera were examined in dilutions 1: 8 and 1 : 32 only for testing the applicability of the IIR in screening the presence of *Toxoplasma* antibody in the population.

It can be concluded from the results summarized in Table 1 and 2 that the IIR as generally less sensitive than the IFAT but it is no less reliable. Only three sera negative in the IFAT were positive in the IIR in the lowest dilution 1 : 2.

DISCUSSION

The most important factor in the successful use of the IIR is the quality of the India ink. From several brands of inks incidentally available only Higgins Black Magic Ink was usuable. When other batches of this mark were tested, it was found that only some of them were suitable. This was further corroborated by extensive testing of 20 samples of different batches of India ink kindly supplied by Faber-Castell. It was found that some batches of ink completely failed to stain the antigen in the IIR and others formed a heavy "fog". On the contrary, other batches stained the *Toxoplasma* zoites nonspecifically in the absence of a specific serum. So far there has been no explanation for the differences. Geck (1977) tried to improve the unsuitable batches of India ink of Hungarian origin by the addition of gelatine. However, we failed using the same procedure when different concentrations of gelatine were tested.

A certain inconvenience of the IIR is the background staining ("fog") which is always present in the area previously covered by the India ink. This "fog" does not hinder the actual evaluation of the reaction, but represents rather a "beauty defect". The fog causes also that the *Toxoplasma* zoites in the negative reaction are covered by the same layer of India ink particles. In such cases the sides of the three-dimension bodies of *Toxoplasma* zoites are more conspicuous during focusing. Only an unexperienced observer could be confused by this. We attempted to remove the "fog", but our attempts were unsuccessful. The fog could be removed from the background but not from the *Toxoplasma* zoites, which thus appeared even more conspicuous.

A certain advantage of the IIR is that the preparations can be stored after observation. Some preparations were preserved for at least 9 months under normal laboratory conditions. The results can be thus reconfirmed as needed.

An intringuing question of the IIR is the role of complement. Without its application the results of the IIR are often uncertain being arbitrary, faint or even negative.

The presence of the complement, however, is not necessary in the IIR for encephalitozoonosis (Waller 1977, Chalupský et al. 1979). In addition Geck (1977) reported the inhibitory effect of complement in the IIR. These results are contradictory. There is no difference in the accuracy of the test if the test sera are fresh or inactivated (deprived of the complement). Substitution of complement by pure amboceptor (SEVAC Prague) in different dilutions has positive but quite unspecific affects. Absorption of the amboceptor by whole *Toxoplasma* bodies had no effect. The same kind of substitution by 1 % gelatine intensifies only the general India ink fog. Experiments aiming at performing the IIR without the complement are in progress.

As far as the theoretical background of the IIR is concerned, it bears some resemblance to the Rieckenberg's (1917) phenomenon in which blood platelets adhered to trypanosomes in the presence of a specific serum. Cookson (1964) mentioned several particulate materials, which adhere to the antigen under similar conditions and discussed the use of this adhesion phenomenon as a specific test in trypanosomiasis and treponemal diseases. Geck (1977) attempted to explain the theoretical basis of the India ink immunostaining. In his opinion the antibodies which attach to the antigen take the particles of India ink with and attach them to the surface of the antigen. For this reason Geck first applied the India ink to a slide with antigen, then

Table 1. Comparison of the IIR and the IFAT using the sera of patients with suspected toxoplasmosis

IFAT \ IIR	Neg.	2	8	16	32	64	128	256	512	1024	2048	Total
Neg.	54	3	—	—	—	—	—	—	—	—	—	57
2	—	1	1	—	—	—	—	—	—	—	—	2
8	1	—	2	2	—	—	—	—	—	—	—	5
16	1	—	8	10	—	—	—	—	—	—	—	19
32	—	—	6	17	17	2	—	—	—	—	—	42
64	—	—	—	22	19	14	—	—	—	—	—	55
128	—	—	2	—	8	16	4	1	—	—	—	31
256	—	—	—	—	1	2	6	5	1	—	—	15
512	—	—	—	—	1	1	5	6	4	1	—	18
1024	—	—	—	—	—	—	1	3	2	2	—	8
2048	—	—	—	—	—	—	—	—	1	—	—	1
Total	56	4	19	51	46	35	16	15	8	3	—	253

Table 2. Comparison of the IIR and the IFAT using randomly selected human sera

IFAT \ IIR	Neg.	8	32	Total
Neg.	157	—	—	157
8	15	27	7	49
32	1	27	46	74
Total	173	54	53	280

added the positive serum and mixed both components. Waller (1977) used the same procedure. Geck's explanation does not seem probable of our experience that the reaction can be performed stepwise, i.e. the treatment of the antigen with serum and India ink need not be simultaneous. The IIR is equally successful when the treatment with serum and complement on one side (followed by washing and drying of the slide) and the treatment with India ink on the other side are performed at different time. When the slides are kept in a refrigerator at 4° C, even the postponement of India ink

application for 24 hours does not hinder the outcome of the reaction. When the slides are left for several days after the application of serum and complement, a gradual deterioration of results can be noticed. Yet after one week some binding of ink is still observable. The results obtained with the slides kept at the room temperature are worse.

A further study is necessary to elucidate the theoretical background of the IIR. However, it seems that some kind of electrostatic charge may be responsible. Similar opinions have been proposed by Cookson (1964) for adhesion phenomena.

The use of the IIR in this time of sophisticated laboratory methods seems old-fashioned. However, the IIR is not only of theoretical but even of practical interest. The IIR in general is less sensitive than IFAT, yet it is so simple, rapid and enexpensive (no fluorescent equipment, no conjugate necessary) that it certainly should be considered a method of a great potential, especially in a large-scale screening and selecting of sera for a later examination by more refined methods. This is confirmed by our experience.

Acknowledgements. Thanks are expressed to Mrs J. Jírová for her skilful technical assistance and to A. W. Faber-Castell for kindly supplying us with the India ink.

ИСПОЛЬЗОВАНИЕ ИММУНОРЕАКЦИИ ПРИ ПОМОЩИ ТУШИ ДЛЯ ДИАГНОЗА ТОКСОПЛАЗМОЗА

Й. Халупски

Резюме. Предлагается использование иммунореакции при помощи туши для диагноза токсоплазмоза. Учитывая несложность метода и нужного лабораторного оборудования, метод особенно удобен для использования в большом масштабе.

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Received 28 August 1980.

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