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INDIRECT HAEMAGGLUTINATION REACTION WITH SARCOCYSTIS DISPERSA ANTIGEN

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Abstract. A description is given of the preparation of antigen from *Sarcocystis dispersa* cystozoites and the procedure of the indirect haemagglutination test (IHA). The antibodies against this antigen were detected in experimentally infected mice from day 20 p.i. (1 : 640). In the following weeks the antibody titres reached the value of 1 : 40,960. The sera of pigs, sheep and horses spontaneously infected with other *Sarcocystis* species reacted with this antigen in low titres only. The bovine sera gave negative reactions even in cases when *Sarcocystis* cysts were present in the muscles of the examined animals. A possible application of IHA for the research and diagnostic purposes is discussed.

The reaction of indirect haemagglutination is still one of the most frequently used serological methods. Its advantages are — in addition to a high sensitivity, ranging this method immediately behind the immunoenzymatic methods — particularly low material requirements, technical simplicity and possibility to reach a high rate of standardization. The IHA reaction has been successfully applied for the diagnosis of many parasitic infections.

In the last ten years, the most commonly used method in the serology of sarcosporidiosis has been the indirect immunofluorescence antibody test (IFAT) (Bordjochki et al. 1972, Tadros et al. 1974, Aryeetey and Piekarski 1976, Piekarski et al. 1978, Černá and Kolářová 1978, Černá et al. 1979, Černá 1980). Lunde and Fayer (1977), Frelier et al. (1977) and Leguia and Herbert (1979) succeeded in using IHA for the diagnosis of sarcosporidiosis in cattle and sheep using *S. cruzi* (*bovicanis*) antigen prepared from heart muscle of experimentally infected calves. The application of IHA for the detection of antibodies against asexual stages of *S. dispersa* in mice was described in our preliminary report (Červa and Černá 1980). One of the prerequisites for a successful application of this method is the possibility to prepare a sufficient amount of absolutely pure antigen, as it is just in case of *S. dispersa*. In experimentally infected laboratory mice masses of parasitic cysts regularly develop in muscle tissues. The cysts can be easily separated from the host tissues.

The surface antigen of *Sarcocystis* cystozoites detected by means of IFAT proved to be genus specific (Tadros et al. 1974, Černá and Kolářová 1978). It was of interest to assess whether the same is true for the soluble antigen in IHA reaction. We have therefore tested the IHA method both for the detection of homologous *Sarcocystis* antibodies and for its application in a heterologous system.

The present paper summarizes the results obtained with the IHA method using *S. dispersa* antigen. Since the method was modified for this purpose, all stages of the procedure are described in details.

MATERIAL AND METHODS

Preparation of parasite suspension. SPF mice strain ICR (Velaz) were infected perorally with the dose of 1 × 10^5 sporocysts of *S. dispersa*. Three months after infection the animals were bled, their muscles were cut into small pieces and digested with trypsin using the method of Erber (1977). After trypsinization, the material was strained through a triple gauze, centrifuged, and the sediment was washed three times in buffered saline (PBS). The final suspension contained 1.8 × 10^9 cystozoites/ml.

Preparation of antigen. The sediment of pure cystozoite suspension was transferred to sterile distilled water and cryolized by five-fold freezing and thawing in a mixture of ice with salt. The cryolizate was centrifuged for 15 min at 1,500 × g and the supernatant was used as antigen. It was either kept in a freezing box at —20 °C or it was lyophilized. The activity of the antigen remained unchanged at least for two years. The concentration of protein nitrogen assessed by Folin's reaction was 19.08 mg/ml.

Preparation of formolized sheep erythrocytes. A volume of 50 ml of sheep blood was collected into a transfusion bottle containing 60 ml of Alsever's solution (20.5 g of glucose, 8.0 g of Na-citrate, 0.55 g of citric acid, 4.2 g of NaCl, distilled water ad 1,000 ml), well mixed and kept at the ice-box temperature for 24 h to several days. The erythrocyte suspension was then washed six times by centrifugation in a surplus of Claus-Jensen (CJ) buffer (KH_2PO_4 1.45 g, Na_2HPO_4 . 12 H_2O 15.29 g, NaCl 5.0 g, distilled water ad 1,000 ml) and suspended to the original volume of 110 ml (the erythrocyte concentration is approximately 20 %).

Washed fresh erythrocytes were poured into a thick-walled bottle (500—1,000 ml) and about 100 ml of glass beads were added. Under constant thorough stirring, 110 ml of 8 % formalin p.a. diluted in CJ buffer were slowly poured in. The contents of the bottle was then vigorously shaken for 30—60 min until the erythrocyte colour turned from light red to dark brown-red which indicated that the fixation was finished. The fixed erythrocytes were left in the bottle with the glass beads and kept in a refrigerator. When stored in this way they can keep their quality for at least one year. The erythrocyte concentration in the fixed suspension was approximately 10 %.

Washing of formolized erythrocytes before use. The fixed erythrocytes in the bottle were well resuspended and the needed quantity was poured into centrifugation tubes. They were then washed 3 times by centrifugation in a surplus of saline and twice in CJ buffer. At 670 × g the erythrocytes sedimented perfectly already after 3 min. The volume of the washed suspension was then adjusted to the original one (= 10% concentration).

Preparation of bovine albumin solution for the dilution of erythrocytes. The lyophilized bovine serum-albumin (TPY 30-98a-72, SEVAC Praha) was diluted to 1% concentration in CJ buffer. One volume portion of this solution (e.g. 5 ml) was mixed with the sediment from two volume portions of formolized erythrocytes (e.g. from 10 ml). The suspension was carefully mixed and incubated at 37 °C for 1 hour. Every 15 min the tubes were shaken. After 60 min the erythrocytes were removed by centrifugation and the supernatant of the adsorbed albumin was used for the dilution of the sensibilized erythrocytes.

The adsorption of bovine albumin with the erythrocytes is not inevitable for the IHA reaction, but we have experimentally verified that the general picture of the reaction was thus improved and the determination of the reaction endpoint was easier.

Adsorption and dilution of sera. The examined and control sera (both negative and positive) were inactivated for 30 min at 56 °C, 0.3 ml volumes were mixed with 0.3 ml of the suspension of washed formolized erythrocytes. The mixtures were incubated at 37 °C and repeatedly stirred. After 60 min incubation the tubes were centrifuged and 0.5 ml of the supernatants were transferred to 4.5 ml of CJ buffer to obtain the basic serum dilutions of 1 : 20.

In a majority of sera examined the adsorption with erythrocytes was unnecessary. However, it was routinely used as it did not decrease the specific titres of sera and prevented the possible unspecific agglutination of erythrocytes.

Sensibilization of erythrocytes. A volume of 0.5 ml of washed, formolized erythrocytes was centrifuged, the supernatant was removed and 1 ml of antigen diluted according to the results of the previous titration was added. The dilution of antigen was performed in the Michaelis' isotonic buffer of pH 5.32. The suspension was well mixed, incubated for 60 min at 56 °C and slightly stirred every 15 min. After the sensitization, the erythrocytes were centrifuged, washed once in CJ buffer and resuspended in 3 ml of CJ buffer with 1 % adsorbed bovine serum albumin. The resulting concentration of the sensibilized erythrocytes was approximately 1.5 %.

Preparation of Michaelis' isotonic buffer

Solution I: 9.714 g CH_3COONa . 3 H_2O
14.714 g Na veronal
H_2O distilled ad 500 ml

Solution II: 8.5 g NaCl
100 ml distilled H_2O
Solution III: 0.1 N HCl
Solution IV: distilled H_2O

The basic solutions I—IV were mixed at the ratio 5 : 2 : 8 : 10.

The prepared solution was poured into 5- to 10-ml ampoules, sterilized in the autoclave and stored at room temperature for an unlimited time period.

Preparation of control non-sensibilized erythrocytes. The procedure was similar to that of sensibilization of erythrocytes. Instead of antigen only pure Michaelis' buffer was used for the incubation.

The techniques of the IHA test. The equipment used was that produced by Dynatech for serological micromethods, i.e. plastic panels 8 × 12 cm with 96 wells with round bottom (U-Form), measuring 0.6 mm in diameter (Microtiter system). Using a special dropper, 0.025 ml of CJ buffer were dispensed to each of the 96 wells. A volume of 0.025 ml of adsorbed serum was transferred with a dilutor from the tube with basic dilution to the first well. The contents was well mixed by at least ten rotations of the dilutor and the dilution in further wells continued in the same manner. Then 0.025 ml of the erythrocyte suspension sensibilized with the antigen were added to each well. Each serum was tested in parallel also with unsensibilized erythrocytes. The well contents in filled panels were then thoroughly mixed by vibration. The reaction was performed in wells filled with 0.05 ml of the mixture of antigen and examined serum in the final dilutions of 1 : 80 to 1 : 163,840. The filled panels were covered and left at room temperature for two hours. After this time or at any time later the reaction could be read.

Evaluation of the reaction

++++ = the whole bottom of the well is evenly covered with a layer of erythrocytes, the margins of the layer are turned up and form an irregular, torn ring

+++ = the whole bottom of the well is covered with an even layer of erythrocytes

++ = the layer of erythrocytes does not cover the whole bottom, there is no marked ring at the periphery (this degree of reaction was evaluated as endpoint of positivity)

+ = at the bottom of the well is a ring of erythrocytes with a light centre, the diameter of which is more than a half of the sediment (this degree of reaction was evaluated as negative)

0 = the erythrocytes sediment in form of a little disc at the well bottom. In an ideal form of the reaction, the disc has no central clearing, in no case the light centre can be larger than a half of the sediment diameter.

Experimental infection of laboratory animals. Mice of ICR strain from SPF breeding Velaz were maintained on pellet diet DOS-IIb (Velaz, Lysá n. L.). A group of 31 males and females weighing 18—20 g was infected perorally with a dose of 1 × 10^5 sporocysts of *S. dispersa* in the suspension prepared from the faeces of *Tyto alba*. At given time intervals, three of the infected animals were sacrificed, their blood was used for our serological examinations and the course of infection in their organs was studied microscopically. Five agnotobiotic adult rabbits giving negative results of IFAT were chosen for the experiments with *S. cuniculi*. They were infected perorally with a dose of 100,000 sporocysts of *S. cuniculi* isolated from cat faeces. The blood was collected from ear vein starting from day 20 after infection at the intervals of 10—20 days.

RESULTS

Application of IHA in a homologous antigen-antibody system

Soluble antigen prepared by the method described above was titrated with the serum of mouse surviving experimental infection with *S. dispersa*. The results of antigen titration are given in Table 1.

The development of a specific antibody response was followed in a group of experimentally infected mice killed at various intervals after infection. The results of examinations are summarized in Table 2.

A conversion of negative serological results to positive ones occurred between days 13 and 20, when relatively high titres were already determined. The titres in individual animals are not strictly related with the length of infection, which indicates that the pathological process develops individually even after a massive inoculum. The highest titre recorded, however, came from a mouse which had survived for 125 days. In the sera of mice inoculated for the preparation of antigens, the antibodies were detectable even in the dilution of 1 : 40,960.

Table 1. Results of titration of *S. dispersa antigen*

	Reciprocals of serum dilutions								
	80	160	320	640	1 280	2 560	5 120	10 240	20 480
Control negative mouse serum Non-sensibilized erythrocytes	o	o	o	o	o	o	o	o	o
Erythrocytes sensibilized with antigen diluted									
1 : 4	+	o	o	o	o	o	o	o	o
1 : 8	+	o	o	o	o	o	o	o	o
1 : 16	+	o	o	o	o	o	o	o	o
1 : 32	o	o	o	o	o	o	o	o	o
1 : 64	o	o	o	o	o	o	o	o	o
Positive mouse serum Non-sensibilized erythrocytes	o	o	o	o	o	o	o	o	o
Erythrocytes sensibilized with antigen diluted									
1 : 4	++++	+++	+++	+++	+++	+++	o	o	o
1 : 8	++++	+++	+++	+++	+++	+++	+	o	o
1 : 16	++++	+++	+++	+++	+++	+++	+	o	o
1 : 32	++++	+++	+++	+++	+++	+++	+	o	o
1 : 64	++++	+++	+++	+++	+++	++	o	o	o

Optimum antigen dilution was 1 : 50 (concentration of protein nitrogen 0.38 mg/ml)

Table 2. Results of IHA reaction in mice sacrificed at various intervals after infection

Day after infection	Reciprocals of serum dilutions		
3.	0	0	0
6.	0	0	0
9.	0	0	0
13.	0	0	0
20.	640	640	640
28.	1 280	1 280	—
38.	640	1 280	0
49.	160	2 560	—
84.	80	320	640
125.	2 560	2 560	10 240

Application of IHA in a heterologous system

In experimental rabbits, the first antibodies in the reaction of IHA with *S. dispersa* antigen were detected as late as on day 114 p.i. in the lowest recorded titre of 1 : 80. Till day 282, when the last blood collection was made, the antibody level increased

to 1 : 320. At the same time interval after infection, the titres of homologous antibodies in mice reached the value of tens of thousands.

In a group of 20 randomly chosen fattened pigs, five animals (25 %) gave positive reactions in the titres of 1 : 80—1 : 640. Microscopical examinations of the muscles could not be carried out.

The haemagglutination test of sera of 40 heads of cattle originating from two localities in Bohemia gave negative results, though in IFAT with *S. dispersa* antigen, 64 % of the examined animals were positive and *Sarcocystis* cysts were very frequently found at microscopical examinations of the muscles of cattle coming from this region. Černá and Merhautová (1981) found sarcosporidia in oesophagi of 84 % of examined animals during microscopical examinations of cattle at Prague abattoir.

The sera of 20 sheep infected with *S. tenella* and another microscopical type of muscle cysts reacted in IHA with *S. tenella* antigen in the titres of 1 : 160 to 1 : 1,280. Positive reactions with *S. dispersa* antigen ranged at the values by 3 to 5 dilutions lower.

In two samples of horse sera supplied by SEVAC Prague for laboratory use, surprisingly high titres (1 : 1,280 and 1 : 2,560) with *S. dispersa* antigen were obtained in IHA.

The control set of sera contained also 20 human sera obtained from the biochemical laboratory. All of these samples were negative.

DISCUSSION

The described modification of IHA stems from the procedures applied earlier for the detection of other protozoan antigens (Červa 1977). Due to the fact that antibodies against Sarcosporidia occur in horse sera, the pure bovine serumalbumin was used as protein vehicle for the suspension of erythrocytes. In contrast to the method used by Lunde and Fayer (1977), in our modifications the erythrocytes are not treated with tannic acid. In this way we avoid a step which can be hardly standardized (quality of tannin) and the possibility of false positive reactions is lesser; however, the antigen concentration must be somewhat higher during the sensibilization of erythrocytes. The final titres in positive reactions in a homologous system are at a corresponding level in both modifications of the reaction.

The experimental application of the method of indirect haemagglutination with *S. dispersa* antigen in tests with sera of animals infected with a homologous parasite species revealed that in this way, specific antibodies can be detected since the third week after experimental infection of the host. The high titre in the positive test persists for the whole period of the host's survival. Surprising is the relatively long period without any antibody response at the beginning of infection. This may be due to the fact that the antibody formation is stimulated only at the time when the parasites pass from the cells of the reticuloendothelial system and liver tissue to the muscle tissue. It is also possible that the antigen prepared from muscle cystozoites is not adequate for the detection of antibodies against the merozoites produced at the early stage of mouse infection. In our opinion, this question is very important from both theoretical and practical reasons.

The examinations of sera from other animal species than mice suggest a certain relation of antigens of *S. dispersa* cystozoites occurring in IHA with that of *S. cuniculi* and some of the species infecting sheep, pigs and horses in our conditions. The levels of antibody titres indicate that this heterologous system detects only the highest levels of homologous antibodies. On the other hand, the negative results obtained with bovine sera show that there is a great difference between the soluble antigen of *S. dispersa* and the antigen of species infecting cattle.

Consequently, we assume that for the time being the IHA reaction with *S. dispersa* antigen can be used mainly for the detection of homologous antibodies in mice with muscle cysts. Only after a detailed study of the antigenic relations with other *Sarcocystis* species it might be used also for the detection of antibodies against the species with which the preparation of a sufficient quantity of pure antigen is still problematic.

The IHA method is sensitive, can be easily standardized and is technically simple in both macro- and micromodifications. Therefore it is an important tool for the detection of immunological response in pathogenetical studies, as well as for the diagnostical detection of specific antibodies.

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НЕПРЯМАЯ ГЕМАГГЛЮТИНАЦИЯ ПРИ ПОМОЩИ АНТИГЕНА *SARCOCYSTIS DISPERSA*

Л. Черва и Ж. Черна

Резюме. Дано описание подготовки антигена из цистозоитов *Sarcocystis dispersa* и метода непрямой гемагглютинации (РНГ). Антитела против антигена обнаружены в экспериментально зараженных мышах с 20-го дня после заражения (1 : 640). В течение следующих недель титры антител достигли уровня 1 : 40 960. Сыворотки свиней, овец и лошадей, спонтанно зараженных другими видами саркоцист, реагировали с этим антигеном только в низких титрах. Бычье сыворотки давали отрицательные реакции даже в тех случаях, когда при микроскопическом исследовании были обнаружены цисты в мышцах животных. Обсуждается возможность применения РНГ для исследования и диагноза.

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Th. Hiepe (Ed.): Lehrbuch der Parasitologie. Band 1. Allgemeine Parasitologie. *VEB Gustav Fischer Verlag, Jena und Gustav Fischer Verlag Stuttgart—New York 1981, 150 pp., 20 Figs., 10 Tables. Price 20 M, 39 DM.*

More than ten years have passed since the 4th edition of the classic textbook of veterinary parasitology by Prof. Dr. A. Borchert and therefore a need of a new text book has become urgent which would comprise data on the latest achievements in this field. The outstanding specialist and experienced tutor, Prof. Dr. Th. Hiepe set about this task with his co-workers, covering the whole subject matter in four volumes. The first volume which has just appeared in coauthorship with Dr. R. Buchwalder and Dr. R. Ribbeck, is devoted to the problems of general parasitology.

The treatise is composed of 8 chapters. Chapter 1 deals with different forms of relationship among organisms. Chapter 2 is concerned with parasitic organisms, discussing the origin of parasitism, its distribution in the animal kingdom, different forms, adaptation and convergence phenomena in parasites, their specificity and life cycles. Chapter 3 provides an account of different categories of hosts with regard to the host specificity and to ontogeny and the mode of transmission of parasites. Chapter 4 examines the parasite-host relationships, dealing with different problems of parasitic infections, harmful influence of the parasite and defensive systems of the host, including resistance and immunity. The subsequent chapter is devoted to ecological aspects of parasitic infections, discussing the problems relevant to biocenoses, parasite populations and the problems of natural focality of diseases. Chapter 6 is concerned with epidemiology and epizootology of parasitoses. The next chapter gives descriptions of different methods used in intravital and postmortem diagnoses and techniques of demonstrating parasites in outer environment. Chapter 8 is the most extensive, covering the problems of parasite control and of control measures in combatting parasitoses. It gives a detailed account of different substances used against parasitic protozoans, helminths and arthropods and of the ways of their application, reviews the control measures taken against molluscs, the intermediate hosts of helminths, and discusses the problem of residues of antiparasitic drugs. A total of 73 formulas of chemical compounds used accompany the text in this chapter, to some of them a list of various radicals appearing in combinations in the structure of the basic formula, is appended. Each chapter is provided with references. At the end of the book there is a subject index.

As a whole the publication is to be favourably received. It is well organized, each chapter containing the necessary scope of information. Though the concepts are briefly explained, they are lucid enough and the definitions of terms are clear-cut. Particularly noted should be the well-arranged text accompanied by not too numerous, but well chosen figures and tables. This first volume of the textbook is intended for a wide circle of readers, for veterinarians, physicians, biologists as well as specialists in agriculture. In these fields it very well serves its purpose. Three subsequent volumes will deal with parasitoses caused by protozoans, helminths and arthropods and are designed primarily to meet the needs of undergraduates in veterinary medicine. The first volume has already proved that the whole set will constitute an original and useful work, whose appearance is to be greeted with satisfaction.

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