DETECTION OF ANTIBODIES TO ENCEPHALITOZOON CUNICULI IN RABBITS BY THE INDIRECT IMMUNOFLUORESCENT ANTIBODY TEST

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Abstract. Indirect immunofluorescence using spores of Encephalitozoon cuniculi as antigen can be used as a reliable detection method for disclosing the presence of encephalitozoan antibodies in rabbits. The method tested on 200 rabbits distinguished clearly non-infected animals (either completely negative or insignificantly positive in sera dilutions 1:2—1:8) from infected animals (positive in dilutions 1:256 or higher).

According to a recent review (Shadduck and Pakes 1971) encephalitozoonosis caused by Encephalitozoon cuniculi (Protozoa: Microsporida) is one of the most common infections of laboratory animals. Its course is mostly chronic and latent, although severe outbreaks with mortality may occur (Vávra et al. 1971, Pattison et al. 1971). The importance of encephalitozoonosis lies in the fact that the disease interferes seriously with experimental work with laboratory rodents in view of its latency (Jones 1964) and that this factor is frequently overlooked. It may, however, be more far-reaching, as infection of 15—76% of laboratory rabbits and 20—50% of laboratory rats and mice has been reported (for references see Shadduck and Pakes 1971).

The present methods of diagnosing encephalitozoonosis are not very satisfactory. In living animals the disease may be recognized by certain clinical symptoms which are, however, present only in severe cases. Provoking a latent infection with cortisone (Innes et al., 1962, Bismar 1970a) or Endoxan (Kaneda 1969) is of little value for practical diagnosis. Detection of parasites in histological sections or injection of tissue into animals or tissue culture are limited to "post mortem" diagnosis and not reliable. Moreover, they are of no use in selecting healthy animals for laboratory experiments.

Stimulated by the lack of reliable methods disclosing encephalitozoonosis in living animals we developed an indirect fluorescent antibody test (IFAT) for the detection of encephalitozoon antibody. Provisional successful results with this method are described in an earlier paper (Chalupský, Bedrník, Vávra 1971).

MATERIAL AND METHODS

The antigen. Spores of Encephalitozoon cuniculi were harvested in mass from tissue cultures of rabbit choroid plexus infected with Encephalitozoon of rabbit or mouse origin and maintained by methods described by Shadduck 1969 and Vávra et al. 1972. When nearly all cells in the culture were infected, they were scraped off the walls of the culture vessels. The infected cells ruptured during this procedure, the spores were released into the medium and were isolated by centrifugation. After washing in phosphate-buffered saline, pH 7.2 by repeated centrifugation (1000 G for 10 minutes),
they were placed either on slides for immediate use or kept at 4°C where they may be stored for at least two months. For application to slides, the sediments were suspended in phosphate buffered saline and diluted to an extent that the average drop from a Pasteur pipette after drying yielded about 50 spores per field of the oil immersion objective. Twelve separate drops were placed on each slide and left to dry completely in the cold room. Slides with the dry spores were fixed by dipping into reagent grade acetone for 20 minutes. After drying a circle was drawn with a greased glass pencil around each drop of antigen. The slides can be either used immediately or stored in a deep-freezer at −20°C where they can be kept for at least two months. Acetone was found to be preferable to 1% formal or methanol for fixation.

The conjugate. Commercial swine anti-rabbit gamma globulin, SWAR g-glob (USOL, Prague) was used. Individual batches of the conjugate were adjusted by dilution with buffered saline, pH 7.1 at a ratio 1:5—1:10 according to their quality.

The microscope. Fluorescence microscope M1-2 (USSR) with filters BS 8-2, FS 1-2, S3S-2 equipped with UV-high pressure mercury lamp DR3 250.

The reaction. Serum from the examined animals was diluted with phosphate-buffered physiological saline 1:2, 1:4, 1:8 for screening, and 1:16 up to 1:128,000 for testing the actual antibody level in positive animals; serum was added by drops to the antigen and kept for 1 hour at 37°C in a moist chamber. After 1 hour the serum was washed off the slides for 10 minutes in tap water and for 5 minutes in physiological saline. Washing was performed in a slide-holder placed on a laboratory shaker specially modified for gentle shaking. After removing excess fluid by partial drying, the conjugate was applied similarly to the antigen and left to react for 1 hour at 37°C in a moist chamber. The conjugate was washed off with tap water and physiological saline. Finally the slide was stained with Evans blue for 5 minutes (stock solution 1:1,000 diluted before use 1:10). The stain was removed by rinsing in physiological saline. After partial drying the slides were mounted in buffered glycerol and read in the fluorescence microscope. The time of application of serum and conjugate can be reduced to 30 minutes if necessary, because the sensitivity of the reaction is lowered only in very high serum dilutions.

Controls. For positive controls we used originally sera from artificially infected rabbits. Later, when the specificity of our IFAT was proved, sera of spontaneously infected rabbits were used as controls. In sacrificed rabbits of the latter group the actual presence of the parasite was confirmed by cultivation and with histological methods. Controls with negative serum and controls without serum were included in each test. The quality of the conjugate was additionally tested with the IFAT for toxoplasmosis.

RESULTS

When using sera of experimentally or spontaneously infected rabbits for the IFAT, the spores show a bright yellow-green fluorescence uniformly distributed on the surface of the spores which appear to be more intense at the margin of the spores (Plate I, a). The developmental stages of the parasite (schizonts and sporoblasts) exhibit a less intense fluorescence (Plate I, b, Plate II, a). The purple background colour of the spore content stained with Evans blue is masked almost completely by the fluorescence. In more diluted sera or in sera with a low positivity the fluorescence is weaker and is concentrated along the spore periphery while the spore interior gives a reddish fluorescence. In still higher dilutions the fluorescence of the spore border is weak and often irregularly distributed. In completely negative reactions the spores have a deep purple colour sometimes with a hardly noticeable brighter rim. The criteria used for a semi-quantitative estimation of the reaction were these:

positive + + : spores with uniform bright yellow-green fluorescence
+ : spores with bright fluorescence on the periphery, central part of the spore without yellow-green fluorescence
negative ± : spores exhibiting irregular or very weak fluorescence on the border. Centre of the spores a deep purple red
Ø : spores dark purple red.

Animals with sera positive in a dilution of at least 1:256 for + + were considered to be positive. Sera with no reaction or those giving a positive reaction only in dilution 1:2 or ± reaction in dilution 1:8 were classified as negative.
We tested 200 rabbits from different sources (laboratory colonies (42), commercial breeding farms (98) and individual small breeders (60). According to their response to the IFAT, the animals could be divided into two categories: 1) negative or insignificantly positive (IFAT negative or weakly positive up to 1:8), 2) positive (IFAT positive for \( \pm \) in dilutions 1 : 256 or higher). These two categories are sharply separated: not one of the 200 rabbits reacted in an intermediary way.

<table>
<thead>
<tr>
<th>Dilution of serum</th>
<th>Number of rabbits</th>
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<tbody>
<tr>
<td>0 — &lt;1 : 8</td>
<td>165</td>
</tr>
<tr>
<td>1 : 16 — 1 : 128</td>
<td>0</td>
</tr>
<tr>
<td>1 : 256 — 1 : 128,000</td>
<td>35</td>
</tr>
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No percentage of infected animals is given for the total of animals examined as the prevalence of the disease varied considerably in the different colonies. Generally at least 50% of rabbits in laboratories are infected and their sera react in dilutions from 1:256 to 128,000. Considerable variation was shown in animals from two commercial rabbit farms. None of the animals from the one farm was infected, while 90% of animals from the other farm showed high levels of anti-encephalitozoon antibodies. No positive animals were detected in small-scale individual breeding.

In some spores the polar filament extruded during the preparation of the antigen. In such spores also the filament showed a bright yellow-green fluorescence in positive sera (Plate II, b, c). Sometimes this fluorescence of the filament persisted even in higher dilutions of the serum by contrast to the fluorescence of the spore itself. In very rare instances extruded spores showed no fluorescence, but the filament showed a relatively bright fluorescence.

In addition we tested antigenic reactivity of spores of several other microsporidia with positive rabbit sera. No significant cross-reactivity was observed when spores of Thelohania opacita (from larvae of Aedes sp.), Stempellia sp. (from Cyclops strenuus), Nosema podiae and Nosema necatrix (from laboratory infected Galleria melonella) and of Nosema bombycis (from the larvae of the silkworm) were used as antigen.

**DISCUSSION**

In our experience the IFAT proved very satisfactory for detecting latent encephalitozoonosis in rabbits. The advantages of this method are these: 1) it can be used in living animals and it requires only a minimum amount of blood or serum, 2) the procedure is easy and quick and the possibility of errors in evaluating the results is limited. 3) IFAT appears to be more sensitive than other diagnostic methods. E.g., in one rabbit positive in a dilution up to 1 : 64,000—120,000 one granuloma only with spores was found in numerous histological sections of the brain. A strain was isolated from the same animal by adding homogenate from the brain tissue to the culture of choroid plexus. However, freshly isolated culture of choroid plexus cells from the same animal remained sterile. This reflects the focal distribution of the parasite in tissues which accounts for difficulties in the diagnosis, in histological sections or by cultivation, unless the incidence of infection is high.

The fluorescence in positive sera of the polar filament in extruded spores shows that not only the spore shell but also the spore content of the Encephalitozoon spore exhibit antigenic properties. Filaments showing a bright fluorescence which persisted even at higher dilutions of positive sera, may have been those in which the spore contents
had just passed through the lumen of the filament. A certain amount of autoimmuno-
fluorescence of the filament may contribute to its relatively intensive brightness.
As regards the low positivity (titres 1:2—1:8) detected in some animals, we can
only speculate on its significance. It seems to be caused by a nonspecific bondage
of antibodies in low dilutions of sera. It may also result from a contact of rabbits with
Encephalitozoon which, for some reason, did not provoke the infection. Similar low pos-
itivity was frequently encountered in human sera (Chalupský et al. 1972).
The results of IFAT were exactly the same irrespective of the origin of the antigen
(mouse or rabbit strain). This confirms Bismanis’s results (1970 b) on the antigenic
similarity between Encephalitozoon of mouse and rabbit origin. This suggests that ence-
phalitozoon of different rodents may belong to the same species or at least that the
chemical composition of their spore walls is very similar.
A provisional conclusion as regards the epidemiology of encephalitozoonosis can be
made from our observations. The absence of infection in rabbits from small household
colonies and from some farms engaged in rabbit breeding only indicates that encepha-
lotitozoonosis is primarily a „laboratory disease” acquired by the rabbit from other
laboratory rodents.

ОБНАРУЖЕНИЕ АНТИТЕЛ ПРОТИВ ENCEPHALITOZOON CUNICULI
У КРОЛИКОВ ПУТЕМ НЕПРЯМОГО МЕТОДА
ИММУНОФЛЮОРЕСЦИЮЩИХ АНТИТЕЛ

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Резюме. Непрямым методом флюоресцирующих антител, при применении спор Encephali-
tozooon cuniculi в качестве антигена, можно обнаружить наличие у кроликов антител против
этих микроскопий. Путем этого метода среди 200 кроликов можно было отличить не-
зараженных животных (с абсолютно отрицательными или незначительно положительными
результатами в разведении сыворотки 1:2—1:8) от зараженных (с положительными
результатами в разведении 1:256 или больше).

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Received 13 February 1973.
Positive immunofluorescence reaction in encephalitozoonosis. Fig. a: a group of mature Encephalitozoon spores showing bright fluorescence (the seemingly different brightness of individual spores is due to their overlapping). Fig. b: chain of sporoblasts (arrow) exhibiting a less conspicuous fluorescence than a group of adjacent spores.
Positive immunofluorescence reaction in encephalitozoonosis. Fig. a: chain of schizonts. Figs. b, c: Spores with extruded filaments.