The serological surveillance of several groups of patients using antigens of *Encephalitozoon hellem* and *E. cuniculi* antibodies to microsporidia in patients

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Abstract. This study was undertaken to attempt to identify correlations between microsporidial seroprevalence data in man, clinical diseases and groups of people at the risk of HIV/AIDS infection. Groups of patients were selected according to the predilection of members of the genus *Encephalitozoon* for nervous and kidney tissue. Female prostitutes and alcohol and intravenous drug abusers were selected as groups at risk of HIV/AIDS infections. A total of 401 samples of human sera were examined for the presence of antimicrosporidial IgG antibodies by ELISA test with a titre of 600 considered borderline positivity. The highest occurrence of antimicrosporidial antibodies was found in the groups of alcohol abusers (16 % from 43 patients), intravenous drug abusers (11 % from 9 patients) and prostitutes (10 % from 80 women) for *E. cuniculi* antigen and in the groups of psychiatric patients (14 % from 44 patients), malaria patients (11 % from 38 patients) and alcohol abusers (7 % from 43 patients) for *E. hellem* antigen. The occurrence of specific antibodies of the six examined diagnostic units (*glomerulonephritis chronica*, *pyelonephritis chronica*, *schizophrenia*, dementia, multiple sclerosis and cerebral stroke) was statistically significant only in patients with *pyelonephritis chronica* and dementia (*p < 0.05*). No cases of microsporidial infection were found among the female prostitutes by parasitological examination, although one case of giardiasis was identified. Sera of patients with high anti-*E. cuniculi* and anti-*E. hellem* antibodies (titres in ELISA of 600 and above) were confirmed by Western blot using *E. cuniculi* and *E. hellem* polypeptides, respectively. These results suggest that the examined patients could show residual antibodies from past or latent infections.

Microsporidia are primitive protozoa which form spores after using an extrusion apparatus for injecting the infective sporoplasm into the host cell. They are obligate intracellular parasites and can infect a wide variety of invertebrates and vertebrates including humans (Canning et al. 1986). Currently, at least four genera of microsporidia (*Encephalitozoon* Levaditi et al., 1923, *Folliculinae orcuttii* Silveira et Canning, 1995, *Trachipleistophora* Hollister et al., 1996, *Enterocytozoon* Desportes et al., 1985) and the collective group *Microsporidium* have been associated with human microsporidiosis, predominantly in immunocompromised individuals. *Enterocytozoon bieneusi* Desportes et al., 1985, first described by Desportes et al. (1985) as a causative agent of chronic diarrhoea in patients with AIDS, is the most commonly diagnosed etiologic agent since it has been also identified in other sites, such as the biliary tree and tracheal and bronchial cells (Canning et al. 1990, Weber et al. 1992b, 1994). *Trachipleistophora hominis* Hollister et al., 1996 has recently been isolated from the skeletal muscle of an AIDS patient in Australia (Hollister et al. 1996).

The genus *Encephalitozoon* is represented by three species: *Encephalitozoon cuniculi* Levaditi et al., 1923, *Encephalitozoon hellem* Didier et al., 1991 and *Encephalitozoon intestinalis* (Cali et al., 1993). *E. cuniculi* was the first microsporidian parasite found in humans (Torres 1927). Until the “AIDS era” there had been only two reports of neurological illness with convulsions caused by *E. cuniculi* in two children not infected with HIV (Matsubayashi et al. 1959, Bergquist et al. 1984a). The number of reported *E. cuniculi* cases in AIDS patients is increasing and *E. cuniculi* was found to be the causative agent not only of intestinal microsporidiosis of AIDS patients (Lukas et al. 1989, Peacock et al. 1991), but also of microsporidian hepatitis (Terada et al. 1987) and peritonitis (Zender et al. 1989). *E. hellem* was first described by Didier et al. (1991) from corneal lesions from three AIDS patients. There are subsequent reports of disseminated infections (respiratory tract, kidney) in AIDS patients (Schwartz et al. 1992, Weber et al. 1993), but it has never been found in the intestine. Both *E. cuniculi* and *E. hellem* organisms are morphologically identical, so immunological, biochemical and molecular-biological techniques must be employed for specific identification. This has raised questions about the identity of *Encephalitozoon* infections diagnosed previously in man. *Septata intestinalis* – described by Cali et al. (1993), currently

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known as *Encephalitozoon intestinalis* (Baker et al. 1995, Hartskeerl et al. 1995), is associated mainly with chronic diarrhoea, but also was found extraintestinally: in the kidney, biliary tree, and nasal mucosa (Orenstein et al. 1992, Weber et al. 1994).

This study was undertaken to find the correlations between microsporidial seroprevalence data and clinical diseases. Groups of the patients were selected according to predilections of the genus *Encephalitozoon* for nervous and kidney tissue. Female prostitutes and alcohol and drug abusers were selected as the risk groups for HIV/AIDS infections, together with the higher possibility of occurrence of *E. cuniculi* antibodies Bergquist et al. 1984b).

**MATERIALS AND METHODS**

**Patients.** Groups of patients in the care of the Hospital České Budějovice, Czech Republic (CR) were selected as follows: 43 psychiatric patients (15 diagnosed as schizophrenia, 10 diagnosed as a dementia, 9 as a reactive depression and 9 as a hysteria); 26 neurological patients (8 with multiple sclerosis, 12 with cerebral stroke, 6 with epilepsy); 43 with chronic renal diseases undergoing hemodialysis (18 with *glomerulonephritis chronic* and 16 with *pyelonephritis chronic*, 4 with polycystic kidney and 5 with diabetic nephropathy); and 58 blood donors. Eighteen neurological patients with multiple sclerosis were from the Neurological Clinic in the South Bohemia, CR. The group of women at the risk for HIV infection (80 females with promiscuous behaviour) was from the Diagnostic Institute in South Bohemia, CR. Sera of Czech patients returning from tropics with malaria (38) and with schistosomiasis (43) were obtained from the Laboratory for Tropical and Opportunistic Parasitic Diseases, Prague, CR. Sera from alcohol (43) and intravenous drug (9) abusers were acquired from the Psychiatric Clinic in South Bohemia, CR.

**Parasitological examination.** Sediment concentrates of stool and urine samples from 80 female prostitutes were examined for microsporidia spores. Several staining techniques – Giemsa, Chromotrope-based technique (Weber et al. 1992a), and Uvitex (van Gool et al. 1993) were utilised.

**Preparation of antigens for ELISA test.** Spores of *Encephalitozoon hellem* and *E. cuniculi* derived from tissue cultures were used as corpuscular antigens for the ELISA test. The *E. cuniculi* strain was originally a mouse isolate, determined by transmission electron microscopy and established for *in vitro* cultivation in monkey kidney cells (Vero E6) at the Institute of Parasitology AS CR, České Budějovice, Czech Republic. Tissue culture of *E. hellem* in Vero E6 cells was obtained by courtesy of Dr. G. S. Visvesvara, CDC, Atlanta, USA. Tissue cultures were cultivated in RPMI medium supplemented by 5% fetal calf serum (FCS) and antibiotics (penicillin, streptomycin, amphotericin). Spores obtained from the medium were isolated by centrifugation at 1000 g for 20 min in distilled water and then in phosphate-buffered saline (PBS). Clean spores were then stored in PBS solution with antibiotics (Antibiotic Antimycotic Sigma) at 4°C.

**ELISA test.** The ELISA test was performed according to the method of Hollister and Canning (1987). The polystyrene microtitration plates (Novogen) were sensitised by introducing 10^5 intact sterile spores per well in coating buffer at 4°C overnight. Sera of patients were examined at first in a screening titre of 200, and then at titres 600 and 1,800. The antigen-antibody complexes were detected by using the peroxidase-conjugated swine anti-human immunoglobulin SwAHuG/Px Sigma and the acetate substrate solution with o-phenylenediamine (OPD) and H_2O_2. The optical densities were read in an ELISA reader at 490 nm. A titre of 600 was considered borderline positive for specific antibodies to microsporidia.

**Western blotting.** SDS-PAGE was performed according to Laemmli (1970) in the Mighty Small II mini-system (Hoefer Sci. Instruments, San Francisco, USA), using 5–17% gradient polyacrylamide gels, containing 375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS, without a stacking gel. Samples of solubilized *E. hellem* and *E. cuniculi* antigens (prepared from 10^9 spores/ml according Visvesvara et al. 1991) were reduced by 0.5% (w/v) dithiotheritol and heated for 5 min at 100°C. After cooling to room temperature, the sulphhydryl groups were alkylated with 0.1 M iodoacetamide (final concentration). The separated proteins were electroforetically transferred to the nitrocellulose paper (according Towbin et al. 1979). The strip containing the molecular weight markers was stained with 0.1% w/v Amido black 10B in methanol : acetic acid : water (25 : 10 : 65). Other strips were blocked overnight with 5% skimmed milk powder in PBS-Tween and incubated for 2 hours at room temperature with sera diluted 1 : 100 and 1 : 300 in PBS-Tween for human sera and rabbit hyperimmune sera, respectively. After washing in PBS-Tween, the strips were incubated for one hour at room temperature with peroxidase-conjugated swine anti-human immunoglobulin SwAHu-Ig P*Px (Sevac) or peroxidase-conjugated swine antirabbit immunoglobulin SwAR P*Px (Sevac). The strips were developed in 50 ml 0.1 M TRIS/HCl buffer at pH 7.6 containing 12.5 mg 3.3-diaminobenzidine with 15 µl of 30% H_2O_2.

**Statistical methods.** GMT – geometric mean of titres was used for the analysis of serological results. The χ-square test was used for the examination of the statistical significance. Associations were judged according to statistical significance at the p < 0.05 level.

**RESULTS**

No cases of microsporidial infections were found among the female prostitutes and only one case of giardiasis was found.

Table 1 summarises the results from 401 human sera examined for antimicrosporidal IgG antibodies. The highest occurrence of antimicrosporoidal antibodies was found in the groups of alcohol abusers (16% from 43 patients), intravenous drug abusers (11% from 9 patients) and prostitutes (10% from 80 women) for *E. cuniculi* antigen and in the groups of psychiatric patients (14% from 44 patients), malaria patients (11% from 38 patients) and alcohol abusers (7% from 43 patients) for *E. hellem* antigen. The highest geometric mean titre
**Fig. 1.** Western blot of *Encephalitozoon cuniculi* polypeptides with 1:300 dilutions of sera from *E. cuniculi* immunized rabbit (1), sera of humans in dilutions 1:100 from female prostitutes (2, 3, 5, 10), nephrological patient – *pyelonephritis chronica* (4), psychiatric patient – hysteria (6), neurological patient – sclerosis multiple (7), intravenous drug user (8), patient with malaria (9), negative human serum (11).

**(GMT)** was found for *E. cuniculi* antigen in the group of intravenous drug (GMT = 417), with alcohol (GMT = 323) abusers ranking second. The group of prostitutes was third (GMT = 297 for *E. cuniculi* antigen, 289 for *E. hellem* antigen).

Table 2 summarises the results of examinations for the specific antimicrosporidial antibodies in various nephrological, psychiatric, and neurological diagnostical units. The occurrence of specific antibodies was statistically significant only in patients diagnosed with *pyelonephritis chronica* and dementia (p < 0.05).

Figs. 1 and 2 show the results of Western blotting. Sera of 12 patients with anti-*E. cuniculi* antibodies (titres in ELISA 600 and above) were examined by Western blot using *E. cuniculi* polypeptides. Nine of them reacted positively with proteins of Mw about 60-67 kDa, one also with a band about 26 kDa; none of which were within the complete range of polypeptides. Similarly, the serum of 13 patients with high anti-*E. hellem* antibodies (titres in ELISA 600 and above) was examined by Western blot using *E. hellem* polypeptides. Five of them reacted positive with proteins of Mw about 60-67 kDa, three of them also with the bands at approximately 15 kDa, 30 kDa and 45, respectively. None of them reacted within the complete range of polypeptides.

**DISCUSSION**

The same patients’ sera (168) were also examined by indirect immunofluorescence test IFAT with *E. hellem* and *E. cuniculi* spores. All sera positive in ELISA test (titres in ELISA 600 and above) were also positive in IFAT with range of titres from 64 to 256. This highest IFAT titre correlated positively with ELISA with a titre of 1,800 (unpublished results).

The serological surveys suggest that specific antimicrosporidial antibodies are relatively common in certain groups of people. Our results agree partially with those of Hollister and Canning (1987). They reported, using an ELISA test with *E. cuniculi* antigen, that no positive serum was found in the sera from 116 healthy people. Positive specific antibodies were found in 6% psychiatric and neurological patients (from 159 patients), in 12% of patients with schistosomiasis (from 175 patients), and in 7% of malaria patients (from 451 patients). Singh et al. (1982) found specific *E. cuniculi* antibodies (by indirect immunofluorescence) in the following groups of patients: in 36% of Ghana malaria patients (from 92 patients), in 43% of Nigerians with tuberculosis (from 89 patients), and in 19% of Malaysian patients with filariosis. Nine-percent of a control group of 111 healthy people were positive, as...
were 4% of a group of 25 animal handlers. A group of patients at risk of HIV infection (homosexual men) was examined by Bergquist et al. (1984b) by indirect immunofluorescence. He found 10 (from 33) men positive for E. cuniculi antibodies. The correlation between E. cuniculi antibodies detection in man by ELISA test, Western blotting, IFAT and peroxidase-antiperoxidase (PAP) tests was studied by Hollister et al. (1991). They suggest that high titres of antibodies, and strong binding of these antibodies to the complete range of immunodominant polypeptides of E. cuniculi in Western blotting, as in the tropical diseases group, are indicative of persisting active infections. In the non-tropical groups (neurological, psychiatric, and renal disorders) they found low titres and weak binding to fewer polypeptides in Western blotting (non-complete range of bands), similar to our results. They suggest that these findings are due to residual antibodies from past or latent infections.

The highly significant occurrence of antimicrosporidial antibodies in patients with pyelonephritis chronica and dementia raises the possibility of E. hellem and E. cuniculi as previously undetected agents of psychiatric and renal diseases. In the case of pyelonephritis chronica, E. cuniculi and E. hellem could be the agents of secondary infections, which pass to the chronically-altered kidney cells. The high occurrence of specific antimicrosporidial antibodies in patients with dementia could be due to poor personal hygiene.

The high occurrence of the antibodies among alcohol and drug abusers could be caused by lower hygiene standard and by possible immunodeficiency in these groups. Our study also shows the high GMT in the group of female prostitutes. None of them were infected with HIV or any other sexually-transmitted disease. All of them were clinically in a good health condition. The detection of microsporidial antibodies may be explained by the high frequency of intimate contacts with a high number of sexual partners, together with a low frequency of condom use and poor personal hygiene. The question is the non-detection of microsporidia in urine and stool samples in this group. The multiplication of parasites to pathogenic levels depends on the immune reaction of hosts and could cause a latent infection.

When the immune status of patients is depressed, this latent infection could turn to the clinical form. The occurrence of specific antimicrosporidial antibodies may be a sign of latent infection, or perhaps only due to simple exposure to spore antigens without current infection. This could be resolved by other studies, including biopsies and polymerase chain reactions in patients with higher levels of antimicrosporidial antibodies.

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