Ultrastructural examination of two cases of stromal microsporidial keratitis

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Two cases with chronic stromal keratitis are described in immunocompetent hosts where the diagnosis was originally thought to be herpetic or adenoviral disease. Light microscopy and ultrastructural examination of corneal tissue by electron microscopy were performed following penetrating keratoplasty (case 1) and corneal biopsy (case 2). Specimens from both cases were analysed for viral identification by PCR. Two different species of Microsporidia were identified. Case 1 represents the fourth reported case of corneal stromal Vittaforma corneae where the spores measured 3.3 \times 1.4 \mu m, arranged in characteristic linear groups of about four to eight. Each spore contained a diplokaryotic nucleus and a single row of ten polar tube coils. By contrast, case 2 is the first reported case of stromal keratitis caused by Trachipleistophora hominis. In this case, spores measured 4 \times 2.4 \mu m, located typically within packets. In this species, the polar tube was arranged as a single row of about 10–13 profiles. Viral DNA could not be amplified by PCR. In conclusion, microsporidial stromal keratitis should be considered in culture-negative cases refractory to medical therapy. As microbiological culture techniques are unsuccessful, diagnosis may only be established following histopathological and ultrastructural examination of corneal tissue.

INTRODUCTION

Microsporidia are tiny obligate intracellular spore-forming protozoa that are common parasites in many animals (Canning & Lom, 1986; Canning, 1993), but until the advent of AIDS were considered to be a rare cause of human infection. To date, up to 150 genera have been described and over 1200 species (Keeling & Fast, 2002). Of these, 14 microsporidian species belonging to seven genera (Entero-zyoza, Encephalitozoon, Nosema, Pleistophora, Vittaforma, Trachipleistophora and Brachiola) and some that are unclassified, have been implicated in human disease (Garcia, 2002).

The most familiar stage in the microsporidial developmental cycle is the spore, as it is more easily detected than the intracellular proliferative stages. Spores are resistant to stress, surviving in the environment for a prolonged period of time. The coiled polar tube present in all microsporidial spores is pathognomonic, with infection occurring by direct invasion of host cells through eversion of this polar tube, allowing passage of spore contents (sporoplasm) into the host cell. A two stage developmental cycle follows – an initial proliferative phase (merogony) followed by a spore-forming phase (sporogony).

Humans infected with microsporidia show an array of symptoms including chronic diarrhoea and wasting syndromes, pneumonitis, bronchitis, nephritis, urethritis, prostatitis, hepatitis, encephalitis, myositis and peritonitis. The most common human symptoms are enteric and associated with HIV infection (Lowder & Wilson, 1995). Ocular manifestations are typically a keratoconjunctivitis, found mainly in immunocompromised hosts (Friedberg et al., 1990; Lowder et al., 1990; Orenstein et al., 1990; Yee et al., 1991; Cali et al., 1991a; Metcalfe et al., 1992; McCluskey et al., 1993). By contrast, stromal keratitis has only been described in six cases and appears to be restricted to immunocompetent hosts. Sclerokeratitis (Mietz et al., 2002) and endogenous endophthalmitis (Yoken et al., 2002) have also been reported.

In this study, we report a further two cases of stromal disease in immunocompetent patients, and outline ultrastructural examination of corneal tissues and species identification.

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**METHODS**

**Case 1.** A 50-year-old male from Cyprus, with negative HIV serology, presented with a right keratitis. There was no history of trauma or contact with animals and a diagnosis of herpes simplex keratitis was made. He was treated initially with topical aciclovir and steroid. The acute inflammation settled but he required a maintenance dose of topical fluoromethalone 0.1 % twice daily to prevent mild episodes of uveitis. Over the next 2 years a yellowish floccular corneal opacity progressed slowly to involve almost the whole cornea, although there was no epithelial defect or vascularization. The visual acuity was reduced to hand movements and a 7-5 mm penetrating keratoplasty was performed. Post-operatively, he was treated with oral aciclovir (400 mg five times daily) and topical dexamethasone 0.1 % 2-hourly. At 1 week following surgery there was a mild anterior uveitis and histology demonstrated the presence of microsporidial spores throughout the cornea. Treatment was changed to topical fumagillin (70 μg ml⁻¹) hourly and prednisolone 1 % q.i.d., and he was given two courses of oral albendazole 400 mg daily, each for 14 days. The inflammation settled rapidly but irreversible graft rejection developed after 6 months. A biopsy of the graft at this stage was negative for microsporidia.

**Case 2.** A 22-year-old Ghanaian gentleman, who had emigrated to the UK in the preceding year, presented with a 12 month history (commencing after his arrival to the UK) of bilateral, episodic photophobia and blurred vision, associated with vitritis and vasculitis. He denied a previous history of contact with animals or agricultural trauma. Following investigations, including negative HIV serology, he was successfully treated for an idiopathic pan-uveitis by systemic immunosuppression with corticosteroids and cyclosporin. Six months later he was noted to have anterior to mid-stromal opacities in his left cornea that were initially thought to be the result of adenoviral infection. Over the next 12 months, these lesions became more confluent (1.5 × 4 mm), with additional epithelial involvement, but no evidence of corneal vascularization or intraocular inflammation. At this stage he was diagnosed with an interstitial keratitis and was commenced on topical corticosteroids and oral aciclovir (400 mg five times a day) with some resolution of his signs. The corneal lesion continued to increase in size, becoming more flocculent in appearance, and after a further 15 months, when the lesion measured 8.0 × 7.5 mm (Fig. 1a), he underwent a corneal biopsy for histopathological investigation and herpes virus PCR, the former demonstrating microsporidial spores. The patient was treated with albendazole 400 mg twice daily in 2 weekly cycles (2 weeks on treatment, 2 weeks off), topical fumagillin (70 μg ml⁻¹), brolene and PHMB 0.02 % every 2 hours. Four months later, he re-presented having discontinued all treatment with a painful and red left eye. The corneal opacity was less dense and this was associated with extensive stromal vascularization (Fig. 1b). There was no view of the fundus, but ultrasonography confirmed a flat retina with some evidence of vitritis. He was recommenced on his anti-microsporidial (fumagillin, albendazole) and immunosuppressives.

**Electron microscopy of tissues.** Biopsy material from both cases was fixed in formaldehyde, post-fixed in osmium tetroxide (1 % w/v), dehydrated in a graded series of ethanol and embedded in Araldite resin. Ultrathin sections were cut from the resin blocks, mounted on copper grids, stained with uranyl acetate and lead citrate and examined in a Philips CM10 electron microscope.

**RESULTS**

**Case 1**

Light microscopy showed abundant microsporidia within the stroma with no evidence of inflammatory infiltrate (Fig. 2a). Electron microscopy confirmed microsporidian spores within the stromal cells. Only the spore stage of development was easily recognizable and these measured about 3.3 × 1.4 μm. Groups of about four to eight spores were generally linearly arranged (Fig. 2b). Internally, spores contained a diplokaryotic nucleus (Fig. 2c) and a single row of ten polar tube coils, with the posterior coils of a smaller diameter (70 nm) than those located more anteriorly (100 nm) (anisofilar arrangement). The host cell endoplasmic reticulum surrounded the spores (Fig. 2d). All of these features (arrangement and size of spores, diplokaryotic nucleus and ultrastructural findings) indicated that the species involved was *Vittaforma corneae* (Silveria & Canning, 1995b). The virus laboratory was unable to amplify herpesvirus DNA from this sample.

**Fig. 1.** Clinical photographs of case 2 (a) after the corneal biopsy and (b) when he re-presented having discontinued all treatment – note the corneal opacity is less dense but there is extensive stromal vascularization.
Case 2

Light microscopy revealed abundant microsporidia located mainly within packets (sporophorous vesicles) in the corneal stroma (Fig. 3a), with very few intact keratocytes and no inflammatory cells. Electron microscopy confirmed that both spores and earlier developmental stages possessed a single nucleus (monokaryotic arrangement) within the remains of keratocytes. Sporophorous vesicles were seen either containing sporoblasts or mature spores alone, or both together (Fig. 3b). Spores measured 4 × 2.4 μm. Internally, the polar tube (that originates at the anterior end of the spore and is coiled towards the posterior end) was arranged as a single row of about 10–13 polar tube profiles, although some displacement of the posterior polar tubes towards the centre of the spore was seen in some spores. The most posterior (two or three) coils were of a smaller diameter (95 nm) than those found towards the anterior (135 nm) (anisofilar arrangement) (Fig. 3c). Early developmental stages showed numerous branched projections from the parasite surface coat that extended into the cytoplasmic remains of stromal cells (Fig. 3d). These ultrastructural features were consistent with the species *Trachipleistophora hominis* (Hollister et al., 1996; Lafranchi-Tristem et al., 2001). PCR failed to amplify herpesvirus DNA.

**DISCUSSION**

Stromal microsporidial keratitis is an uncommon manifestation of microsporidiosis, with the more common keratoconjunctivitis being most frequently associated with patients with AIDS. Early cases from the USA (Friedberg et al., 1990; Lowder et al., 1990; Orenstein et al., 1990; Yee et al., 1991; Cali et al., 1991b; Metcalfe et al., 1992; McCluskey et al., 1993) and the UK (Hollister et al., 1996) and Australia (Lafranchi-
Tristem et al., 2001) identified the microsporidian species to the genus *Encephalitozoon*, and it was later recognized as a new species, *Encephalitozoon hellem* (Didier et al., 1991; Schwartz et al., 1993; Hollister, 1993a). Other species involved include *Encephalitozoon* (*Septata*) intestinalis (Lowder et al., 1996), possibly *Encephalitozoon cuniculi* (Aarons et al., 1994, Hollister et al. 1993b) and *T. hominis* (Field et al., 1996).

Fig. 3. (a) Light micrograph showing the microsporidian *T. hominis* within the corneal stroma of case 2 (toluidine blue-stained resin section). Note by contrast to *V. corneae* in Fig. 2, the spores are in packets (sporophorous vesicles). Electron micrographs illustrating (b) a sporophorous vesicle of *T. hominis* containing a number of mature spores and earlier sporoblast (arrow) stages; (c) profiles of the polar tube of spore cytoplasm – note the posterior coils (arrows) are of a smaller diameter than the more anterior ones (anisofilar arrangement), and the prominent ring of lucent fibres in the wall of the polar tube and (d) the merogonic stage of *T. hominis* showing numerous short branched projections of the surface coat extending into the remains of the cytoplasm (arrows) of the infected stromal cell.

There are six previous reports of stromal microsporidial disease. The first case was of an 11-year-old boy from Ceylon who presented with a scarred and vascularized cornea (Ashton & Wirasinha, 1973) – the organism being named as *Microsporidium ceylonensis* (Canning & Lom, 1986). Subsequent reports were from a 26-year-old woman from Botswana (Pinnolis et al., 1981) infected with *Microsporidium africanum* (Canning & Lom, 1986); a 45-year-old man from South Carolina, who had travelled to the Caribbean and Central America (Davis et al., 1990; Shadduck et al., 1990), infected with *Nosema corneum* (subsequently renamed *V. corneae*) (Silveria & Canning, 1995b); a 39-year-old man from Ohio with corneal ulceration, infected with *Nosema ocularum* (Cali et al., 1991a); a 67-year-old man from Mexico infected with *Nosema algera* (Visvesvara et al., 1999; Font et al., 2000) and a 65-year-old Caucasian with corneal
stromal *V. corneae* (Font et al., 2003). Further work involving electron microscopy on the original material surviving from the first stromal case (Ashton & Wirasingha, 1973; Canning et al., 1998) was unable to establish a generic placement for the organism involved, but attention was drawn to the similarities between *M. ceylonensis* and *Nosema* sp. described from the cornea of the case from Botswana (Pinnolis et al., 1981).

Case 1 is the fourth reported case of *V. corneae* in human disease. *V. corneae* was first described (as *N. corneum*) in a stromal biopsy from a non-HIV-infected man with an 18 month history of central disciform keratitis, recurrent patchy infiltration of the anterior stroma and iritis (Shadduck et al., 1990). The patient was treated with topical steroids and broad-spectrum antibiotics, but ultimately required a corneal transplant. Initially identified as a new species, *N. corneum*, infection with this organism was subsequently established in athymic mice and the new taxonomically significant features found warranted placing this organism within a new genus *Vittaforma*, as *V. corneae* (Silveria & Canning, 1995a). In the second reported case of *V. corneae* infection (Davis et al., 1990; Shadduck et al., 1990; Deplazes et al., 1998) dual microsporidial infection was detected in a patient with AIDS – *E. hellem* in the sinusasal aspirate, and *V. corneae* in urine (Deplazes et al., 1998), indicating that *V. corneae* is capable of dissemination and survival in deep tissues, at least in the immunocompromised host (Silveira et al., 1993). A third case involving *V. corneae* infection of the corneal stroma in an immunocompetent 65-year-old man has recently been published (Font et al., 2003).

*T. hominis* is a microsporidium that is usually associated with muscular disease (Hollister et al., 1996; Field et al., 1996; A. Curry, unpublished data). Only one previous report of *T. hominis* describes keratoconjunctivitis in a patient with AIDS (Field et al., 1996), and case 2 represents the first report of stromal keratitis in a patient with negative HIV serology. Sources of *T. hominis* infection are uncertain. This parasite is similar to microsporidian parasites (genus *Pleistophora*) found in the muscle of some fish (Cheney et al., 2000). Infected fish could contaminate water or induce infection after consumption, if inadequately cooked. Future molecular studies of microsporidian species found in fish may identify parasites with features of the genus *Trachipleistophora* and indicate a possible source of human infection. An alternative route of infection may be biting insects, as *T. hominis* has a close phylogenetic relationship with microsporidia of the genus *Vavraia*, which are parasites of mosquitoes (Cheney et al., 2000). *Nosema algerae*, which has been identified in one human case involving corneal infection (Visvesvara et al., 1999), is known to be a parasite of anophelene mosquitoes (Trammer et al., 1999). Whatever the source of infection in case 2, it is possible that establishment of the infection was aided by systemic immunosuppressive therapy involving corticosteroids and cyclosporin.

Although apparently localized to the eye, both *T. hominis* and *V. corneae* have the potential for dissemination to other sites and organs, so far, at least, only in the immunocompromised. Both previous *T. hominis* cases originated from Australia, were detected in individuals in the late stages of HIV infection and mainly involved infection of skeletal muscle (Yee et al., 1991; Field et al., 1996; A. Curry, unpublished data). The first case involved infection of skeletal muscle and corneal epithelium, but spores were also detected in sputum (Field et al., 1996). In the second case, infection was detected in skeletal muscle and myocardium (A. Curry, unpublished data). The stromal involvement seen here (case 2), rather than the epithelial infection seen previously, was also suggestive that *T. hominis* was not restricted to specific cells and should be considered as a possible multiorgan pathogen (Field et al., 1996).

Sources of infection of these two parasites for humans are uncertain but may involve animals (Curry, 1999). *V. corneae* spores have been identified in water supplies (Dowd et al., 1998) and spore-contaminated water may be a source of human infection, particularly in the immunocompromised. In case 1 described here, the patient had negative HIV serology, but had received topical immunosuppression with steroid and antiviral treatment for clinically diagnosed herpes simplex infection (not laboratory proven). It is possible that this immunosuppressive steroid treatment may have exacerbated pre-existing microsporidial infection or facilitated *de novo* infection (Dowd et al., 1998).

Routes of microsporidial infection in the eye are unclear. Abrasions into which microsporidial spores are inoculated would seem to be an obvious route, particularly in immunocompetent individuals. *M. ceylonensis* may have been introduced into the eye this way, as the boy had been gored in the right eye by a goat approximately 6 years previously (Ashton & Wirasingha, 1973). However, in patients with AIDS with disseminated microsporidial infection, ocular infection may be acquired by reverse passage from a respiratory source through the lachrymal canaliculi and nasolachrymal ducts that drain secretions from the eyes into the nasal sinuses (Curry & Canning, 1993). Equally, infection may spread from the eyes into the respiratory tract in these patients. It is possible that several factors are required to establish microsporidial infection in the eye. Some form of immunosuppression [either by primary (hereditary) HIV infection, or by use of topical corticosteroids or other forms of systemic immunosuppression] is certainly one factor. The other may be a slightly lower temperature (Cali et al., 1998). If some of the microsporidian parasites found in humans are from poikilothermic (cold-blooded) animals, then the slightly lower temperature of the eye (because of its exposed position) may allow opportunistic parasite development to become established, causing symptoms, whereas, development in deeper (and warmer) tissues could curtail parasite development (Trammer et al., 1997).

Laboratory diagnosis may be difficult because of the small size of these parasites, their intracellular location and poor staining properties (particularly of the proliferative stages) with histological stains. Diagnosis can be made by identifying microsporidian spores (from faeces, urine, secretions or...
tissue smears) or tissue biopsy (Garcia, 2002). Spores may be identified in specimens by commercially available modified trichrome stains, Giemsa and optical brightening agents such as Calcofluor white. In histological specimens, Gram stains, PAS, silver stains and Grocotts are useful, but electron microscopy is the method of choice for tissues, as it allows positive identification and can determine the species involved. Neuter, more sensitive molecular techniques, such as real-time PCR (Wolk et al., 2002) and use of antibodies, such as immunofluorescence and monoclonal antibody linked ELISA (Bouladoux et al., 2003), are also being explored. Molecular identification is available for the most common microsporidial infections found in humans (Franzen & Muller, 1999), but for less common species, electron microscopy should be seen as essential. Efficacy of antimicrobial agents may be evaluated by in vitro culture of several microsporidia, but special tissue culture techniques are necessary, as microsporidia in general are difficult to grow (Lafranchi-Tristem et al., 2001; Silveria & Canning, 1995b; Visvesvara, 2002).

Management of corneal microsporidiosis is difficult. In early cases the eye was lost before diagnosis was made, or a penetrating keratoplasty failed (Ashton & Wirasinha 1973; Pinnolis et al., 1981). There have been isolated reports of response of cases of keratoconjunctivitis to topical fumagillin (50 μg ml⁻¹) (Rosberger et al., 1993; Diesenhouse et al., 1993) and oral albendazole (400 mg twice daily; a benzimidazole related to thiabendazole) (Gritz et al., 1997; Blanshard et al., 1992). Nevertheless, laboratory determination of the microsporidian species involved in human disease is important, as some species, such as Enterocytozoon bieneusi, are not eliminated by treatment with albendazole (Blanshard et al., 1992; Dieterich et al., 1994). It is, however, uncertain whether medical treatment is able to stabilize corneal stromal disease. Debubking microsporidial infection by penetrating keratoplasty with cryotherapy to the recipient edge has been performed successfully with no recurrence of disease (Davis et al., 1990). A full thickness graft is recommended as opposed to lamellar graft due to increased risk of recurrence in the residual tissue at the deep interface if a partial thickness graft is performed (Font et al., 2003).

In summary, microsporidial stromal keratitis is an emerging cause of chronic keratitis refractory to medical therapy and should be considered in all culture-negative cases. Corneal biopsy, and histopathological and ultrastructural examination are vital to establish the diagnosis.

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