Persistence of acanthamoeba antigen following acanthamoeba keratitis

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Abstract

***Aim*—To investigate the hypothesis that persistent corneal and scleral inflammation following acanthamoeba keratitis is not always caused by active amoebic infection but can be due to persisting acanthamoebic antigens**

***Methods*—24 lamellar corneal biopsy and penetrating keratoplasty specimens were obtained from 14 consecutive patients at various stages of their disease and divided for microscopy and culture. Histological sections were immunostained and screened for the presence of *Acanthamoeba* cysts by light microscopy. Cultures were carried out using partly homogenised tissues on non-nutrient agar seeded with *E coli*. Clinical data were obtained retrospectively from the case notes of these patients.**

***Results*—Of the 24 specimens, 20 were obtained from eyes that were clinically inflamed at the time of surgery. *Acanthamoeba* cysts were present in 16 (80%) of these 20 specimens, while only five (25%) were culture positive. *Acanthamoeba* cysts were found to persist for up to 31 months after antiamoebic treatment.**

***Conclusion*—These findings support the hypothesis that *Acanthamoeba* cysts can remain in corneal tissue for an extended period of time following acanthamoeba keratitis and may cause persistent corneal and scleral inflammation in the absence of active amoebic infection. In view of these findings, prolonged intensive antiamoebic therapy may be inappropriate when the inflammation is due to retained antigen rather than to viable organisms**

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First described in 1973,[1] acanthamoeba keratitis is a severe and potentially sight threatening ocular infection, characterised by a waxing and waning course of chronic progressive corneal inflammation and ulceration.[2] Anterior scleritis, and more rarely posterior scleritis, is a well known component of acanthamoeba keratitis.[2–4] Since the first successful medical treatment of acanthamoeba keratitis,[5] the management and visual prognosis of this condition has improved dramatically and the requirement for penetrating keratoplasty reduced markedly.[6 7] However, chronic keratitis and scleritis remain features of the infection despite successful antiamoebic therapy. Persistent corneal and scleral inflammation may be a secondary effect of the corneal infection by the protozoan. Blackman *et al*[8] noted that while intact amoeba were not surrounded by inflammation, necrotic organisms were surrounded by an intense cellular reaction and suggested that the amoebic cyst walls and the cytoplasm of the trophozoites could be the primary site of antigenicity. Several reports[2–4 9–12] were in consistent agreement that intact living acanthamoebal organisms incite little inflammatory reaction.

The rationale of therapy for persistent corneal and scleral inflammation, which occasionally leads to ocular perforation, depends on the nature of its stimulus. We reviewed histopathological and microbiological evidence for the presence of amoebic cysts and viable amoebae in corneal biopsies obtained from patients with acanthamoeba keratitis who had the disease for different periods before biopsy. We correlated these data with the clinical status of the patients to investigate the hypothesis that some cases of persistent corneal and scleral inflammation can be due to an inflammatory response to persisting acanthamoeba antigens rather than to viable amoeba.

Methods

A consecutive series of patients who underwent penetrating keratoplasty or lamellar corneal biopsy for acanthamoeba keratitis between November 1990 and April 1997 were identified from the surgical diary. Clinical data, including the duration of symptoms and the duration of antiamoebic treatment before surgery, were obtained from the case notes. The eye was considered inflamed at the time of surgery if there were signs of scleritis, corneal inflammation, or required oral or frequent topical immunosuppressive treatment to control inflammation. The lamellar corneal biopsies were obtained using a 3 mm skin trephine to demarcate an area off the visual axis or, where debridement of necrotic tissue was necessary, a diamond blade was used to demarcate the area. The demarcated lamellar button was then removed using a Paufique knife. All specimens were bisected and equal halves were sent for microbiological culture and histopathological analysis.

Microbiological culture was performed by an ocular microbiologist (MM). Using a Wheaton tube, the specimen for culture was processed to disrupt the integrity of the tissue but not to completely homogenise the tissue. The fibrous component of the tissue was then plated onto non-nutrient agar seeded with live *Escherichia coli* and the plates were incubated at 37°C for 72 hours. Using binocular microscopy, acanthamoebal trophozoites were identified visually by the presence of vacuoles within

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Table 1 Clinical details of each patient and the results of microbiologic culture and histological analysis of each biopsy specimen

Patient No	Duration of symptoms before diagnosis (weeks)	Specimen No (type)	Antiamoebic therapy at the time of tissue biopsy	Duration of antiamoebic therapy at time of tissue biopsy (months)	Immunosuppression treatment at the time of tissue biopsy	Indication for tissue biopsy	Microbiology	Histopathological analysis		Size of tissue used for histological analysis
							Culture	Cysts	Trophozoites	
1	4	1 (PK)	PR+PHMB	10	TS, NSAID	Persistent Inflammation	Negative	Present	Not present	½ corneal button
2	16	2 (PK)*	PR+PHMB	21	Nil	Scar excision	Positive	Present	Not present	½ corneal button
3	20	3 (PK)	PR+PHMB	5	TS	Persistent Inflammation	Negative	Present	Not present	½ corneal button 8 mm
		4 (PK)	PHMB	9	TS, NSAID	Persistent Inflammation	Negative	Present	Present	½ corneal button 9 mm
		5 (LCB)	PHMB	12	TS	Persistent Inflammation	Negative	Present	Present	3×2×0.5mm
		6 (PK)	PHMB	13	TS	Persistent Inflammation	Negative	Present	Present	½ corneal button
4	10	7 (LCB)	PR	1	TS, NSAID	Excision of Necrotic tissue	Negative	Present	Not present	4×2×1mm
		8 (PK)	PR	4	TS,SS, SC	Corneal perforation	Positive	Present	Present	½ corneal button 7.5 mm
5	17	9 (PK)	PR+PHMB	16	TS, SS	Persistent Inflammation	Positive	Not present	Not present	½ corneal button
6	4	10 (LCB)	PR+PHMB	7	TS	Persistent Inflammation	Positive	Not present	Not present	"Small fragment"
		11 (PK)	PR+PHMB	8	TS	Descemetocoele	Negative	Present	Not present	½ corneal button
7	24	12 (LCB)*	PR+PHMB	11	Nil	Scar excision	Negative	Present	Not present	½ corneal button 0.25 mm
8	16	13 (LCB)	PHMB	7	TS	Persistent Inflammation	Negative	Not present	Not present	"Small fragment"
		14 (PK)	PR+PHMB	16	TS, NSAID	Corneal perforation	Positive	Present	Not present	½ corneal button 4 mm
9	0.5	15 (LCB)	CH+PHMB	6	TS, NSAID	Persistent Inflammation	Negative	Not present	Not present	1.5×1×0.5mm
10	16	16 (LCB)	CH	2	Nil	Persistent Inflammation	Negative	Present	Not present	2 of 1×1×0.5mm
		17 (PK)	CH+HX	4	TS, SS, SC	Corneal perforation	Positive	Present	Not present	½ corneal button6×7 mm
11	6	18 (PK)	PR	4	TS,NSAID, SS	Corneal perforation	Negative	Present	Not present	¼ corneal button 6×7 mm
12	3	19 (LCB)	PR	10	TS	Persistent Inflammation	Negative	Present	Present	Not recorded
		20 (LCB)	PHMB	21	TS	Persistent Inflammation	Negative	Present	Not present	Not recorded
		21 (PK)*	PHMB	31	TS	Scar excision	Negative	Present	Not present	½ corneal button 7 mm
13	2	22 (LCB)	PR+PHMB	2	TS, NSAID	Persistent Inflammation	Negative	Present	Not present	2mm fragment
		23 (LCB)	PR+PHMB	3	TS, NSAID	Persistent Inflammation	Negative	Present	Not present	1mm fragment
14	4	24 (LCB)*	PR+PHMB	21	TS	Scar excision	Negative	Present	Not present	Not recorded

*Specimens obtained from patients with no evidence of clinical inflammation at the time of tissue biopsy.
PK = penetrating keratoplasty; LCB = lamellar corneal biopsy; PR = propamidine isethionate; PHMB = polyhexamethyl biguanide; CH = chlorhexidine; HX = hexamidine; TS = topical steroids; NSAID = systemic non-steroidal anti-inflammatory drugs; SS = systemic steroids (prednisolone); SC = systemic cyclosporine.

the trophozoites. If there was no growth at this stage the plates were incubated for a further 72 hours at 30°C, then if necessary at room temperature, and were not finally discarded for 3 weeks.

The presence of *Acanthamoeba* cysts was identified by an ocular pathologist (IC) using a standard indirect immunoperoxidase technique with rabbit polyclonal IgG purified fraction antiacanthamoeba antibody. An avidin-biotin complex method was used for the immunostaining. Paraffin processed sections (5 μm) were rehydrated through xylene and a series of alcohols, blocked for endogenous peroxidase with 0.5% hydrogen peroxide for 25 minutes, washed in running water, trypsinised (37°C, 10 minutes, 1 mg/ml in TRIS buffered saline, pH 7.8), washed in running water, blocked with 10% normal swine serum in phosphate buffered saline (PBS) of pH 7.3 for 30 minutes, and then incubated overnight at 4°C with a 1 in 2500 dilution of a rabbit antiacanthamoeba polyclonal antibody (kindly supplied by Dr Simon Kilvington, Department of Microbiology and Immunology, Medical Sciences Building, Leicester, UK). Sections were then washed in PBS and incubated with a biotinylated anti-rabbit immunoglobulin antibody (1 in 300 dilution; Dako Ltd, Ely, UK) for 45 minutes at room temperature and again washed in PBS. Following a 45 minute incubation at room temperature with avidin-biotin complex (Dako), bound antibody was visualised using 0.6 mg/ml diaminobenzidine (DAB; Sigma, Poole, UK) incubated with the sections for 15 minutes at room temperature. A Mayer's haemalum counterstain was applied and sections dehydrated before mounting in DPX under glass coverslips. *Acanthamoeba* cysts were stained dark brown and light microscopy was used to detect the presence of the cysts in the tissue.

Results

Twenty four corneal tissue specimens from 14 patients were examined as some patients had more than one corneal tissue biopsy at different stages of the infection. In 10 of the 14 patients initial diagnoses of acanthamoeba keratitis were based on positive cultures or histological identification of *Acanthamoeba* in epithelial or corneal biopsy specimens. The four remaining patients (Table 1; patient nos 6, 7, 11, 12) were initially diagnosed on the basis of clinical symptoms and signs. *Acanthamoeba* cysts were subsequently present in at least one of the corneal tissue samples taken from each of these four patients. The 14 patients had been symptomatic for 0.5–20 weeks (mean 10 weeks, SD 8 weeks) before commencement of antiamoebic treatment. A brief clinical description of each patient including duration and type of antiamoebic therapy and immunosuppression used, indications for tissue biopsies, clinical inflammatory status at the time of tissue biopsy, is given in Table 1. Antiamoebic treatment used included polyhexamethyl biguanide (PHMB), propamidine isethionate, chlorhexidine, and hexamidine. These were used in combination in some patients.

Table 2 Results of microbiological culture and histological analysis of each specimen in relation to the type of biopsy and clinical inflammation at the time of tissue biopsy

		Eyes inflamed at the time of surgery		*Eyes uninflamed at the time of surgery*		
		Penetrating keratoplasty	*Lamellar corneal biopsy*	*Penetrating keratoplasty*	*Lamellar corneal biopsy*	*Total*
Cysts present	Culture positive	3	0	1	0	4
	Culture negative	6	7	1	2	16
Cysts absent	Culture positive	1	1	0	0	2
	Culture negative	0	2	0	0	2

Twelve of the tissue biopsies were in the form of corneal buttons obtained from penetrating keratoplasties and a further 12 were fragments of corneal tissues obtained from lamellar corneal tissue biopsies. The sizes of the tissue biopsies used for histological analysis, when included in the histological report, are given in Table 1, as are the results of the microbiological culture and histopathological analysis for each specimen.

Acanthamoeba cysts were identified in 20 of the 24 specimens of which four were also culture positive (Table 2). Two specimens were culture positive but negative on immunostaining. Of the 20 specimens obtained from eyes that were clinically inflamed at the time of surgery, 16 were positive for *Acanthamoebic* cysts on immunostaining but only five were culture positive. Of the five culture positive eyes, three were obtained at the time of repair of corneal perforation (Table 1: specimens 8, 14, 17) and a further two specimens (Table 1: specimens 9, 10) were obtained from tissue debridement in a persistently inflamed eye. Of the four eyes which showed no signs of clinical inflammation, all were positive on immunostaining for cysts and one was still culture positive.

In the histological preparation, while presence of the acanthamoebic cysts were easily identified by the dark brown immunostain, its internal structure, and thus the viability, of the cysts could not be determined with confidence. While some cysts appeared to have no internal content, this could not be reliably assessed in the histological sections because of the presence of only a part of the cyst in the 5 μm section in many instances. Cyst numbers were variable within individual specimens and counts were therefore not performed. Trophozoites were seen in the histological preparation in only five of the 20 specimens (Table 1; specimens 4, 5, 6, 8, 19) obtained from eyes that were clinically inflamed and only one of these were culture positive. The culture positive specimen (specimen 8), as mentioned previously, was obtained at the time of repair of corneal perforation. Specimens 4, 5, and 6 were obtained from the same patient at different stages of the infection. In specimen 4, heavy infiltration of acanthamoebal trophozoites was seen in the histological sections, subsequent biopsies (specimens 5 and 6) only revealed scattered organisms in trophozoite form. Specimen 19 was obtained from tissue debridement in a persistently inflamed eye. Only scattered trophozoites were found in specimens 8 and 19. No trophozoites were seen in the specimens obtained from the uninflamed eyes.

Discussion

Although acanthamoeba keratitis is now widely reported, it remains a rare cause of corneal infection and, like previous reports examining corneal stromal tissue in acanthamoebic keratitis,[11 12] a small sample size is inevitable. However, our results highlight several findings of interest. Our finding that acanthamoebic cysts were present in the majority of clinically inflamed eyes (80%, 16/20), of which only 25% (5/20) were culture positive for amoeba, is consistent with our hypothesis that persistent corneal and scleral inflammation is not always caused by active amoebic infection but can be due to an inflammatory response to persisting amoebic antigens. As previous reports suggest,[3 4 9–12] the amoebic cyst walls and necrotic organisms are likely to be the site of antigenicity.

Although it is conceivable that our negative cultures could be false negatives, using a stringent clinical and laboratory diagnostic criteria for diagnosing acanthamoeba keratitis, a previous paper[6] with data from the same microbiology laboratory with a large experience in *Acanthamoeba* culture found a positive yield of 50% (33/66) on epithelial biopsies, 55% (11/20) on corneal stromal tissue biopsies, and 60% (9/15) on corneal buttons. Furthermore, our clinical experience as well as that of Holland *et al*[10] of successfully treating post-acanthamoeba keratitis associated corneal and scleral inflammation with topical and/or systemic immunosuppressive therapy conform with the above hypothesis.

Nine specimens were found to be either culture positive or to harbour *Acanthamoeba* in trophozoite form. It is possible that active amoebic infection indicated by presence of trophozoites and/or culture positivity, may be present in these nine specimens. However, the corneal and scleral inflammation may not be due to the active infection by the protozoa per se but to the concurrent presence of necrotic organisms and amoebic cysts walls. Various reports[2–4 9–12] have found that intact cysts and active trophozoites incite minimal inflammatory reaction in the corneal tissue. It has also been suggested that intact living organisms may have the ability to mask their antigens from the cellular immune response.[8 9]

However, contrary to our hypothesis that persistent inflammation is due to the continued presence of amoebic antigens in the amoebic cyst walls, all four of the corneal tissue specimens obtained from eyes which showed no signs of clinical inflammation were found to harbour cysts in the corneal tissue. One of these specimens remained culture positive.

The absence of clinical inflammation in these eyes in the presence of cysts may be due to the loss of antigenicity of the cysts with time, a relatively deficient host immune response or the re-establishment of the corneal immune privilege.[13 14] In these specimens the cysts were present for up to 31 months of antiamoebic treatment respectively. The prolonged presence of acanthamoebic cysts in these corneal tissue specimens, suggests that, like infection with filamentous fungi, amoebic cysts take a long time to clear from corneal tissue. Four of the eyes that were clinically inflamed showed no evidence of amoebic cysts, two of which were culture positive. It is probable that these represent false negatives for histology. Three of these specimens (Table 1; specimens 13, 15) were small fragments of corneal tissue. However, no cysts could be found on further immunostaining and microscopy of new sections on all corneal tissues that were initially reported to be negative. This finding suggests that only very few cysts may have been present and were not present in the sections examined.

While the nature of the stimulus to persistent corneal and scleral inflammation following acanthamoeba keratitis would benefit from further investigation, our finding demonstrates that this may be a result of acanthamoebic cysts persisting in the corneal tissue for an extended period of time and causing an inflammatory response unrelated to the persistence of viable and active amoebae. In some cases with persistent inflammation, no evidence of amoebic antigen was present suggesting that other mechanisms may have been important—for example, chronic inflammation alone may be important. It would be interesting to show the presence of a persisting anti-acanthamoeba immune response in future studies.

Our finding presents a diagnostic dilemma as, at present, there is no non-invasive way of distinguishing viable from non-viable cysts in corneal tissue and invasive methods, such as corneal biopsy, are limited in their extent and may not identify trophozoites or cysts present in the remaining tissue. It is possible that in vivo confocal microscopy could be developed, with vital staining for viable cysts, to distinguish between inflammation and the presence or absence of viable amoebae. Until such methods are available the levels of antiamoebic treatment and anti-inflammatory therapy have to be based on clinical intuition and a realistic evaluation of the information derived from biopsies. Despite these limitations an understanding of these two disparate causes of persisting corneal and scleral inflammation following acanthamoeba keratitis should assist in the treatment of this condition; prolonged intensive antiamoebic therapy may be inappropriate, increasing the morbidity of the disease, when the keratitis is due to retained antigen rather than to viable organisms.

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