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DIAGNOSIS AND CYTOPATHOGENICITY OF *ACANTHAMOEBA*, *VAHLKAMPFIA* AND *HARTMANNELLA* IN CORNEAL TISSUE

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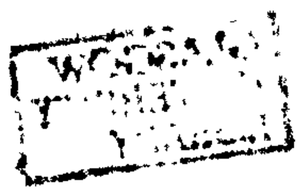

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Accompanying Material

CD-ROM of time lapse videos illustrating the cytopathic effect produced by Acanthamoeba *castellanii* on cultured keratocytes and corneal epithelial cells and by V-EYE and H-EYE on keratocytes.

LIST OF PUBLICATIONS

Aitken, D., Hay, J., Kinnear, F.B., Kirkness, C.M., Lee, W.R. & Seal, D.V. (1996) Amoebic keratitis in a wearer of contact lenses due to a mixed *Vahlkampfia* and *Hartmannella* infection. *Ophthalmology* **103,** 485-494.

Hay, J., Kinnear, F.B., Kirkness, C.M. & Seal, D.V. (1995) Acanthamoeba keratitis: laboratory diagnosis, characterisation of protozoa and treatment. *SCIEH Weekly Report* **28 April 1995 Volume 29 No.95/17,** 90-91.

Kinnear, F.B., Hay, J. & Dutton, G.N. (1995) Difficult ocular infections: the experience of one eye institute. *British Journal of Optometry & Dispensing* 3, 49-57.

LIST OF ABBREVIATIONS

AC_a	axenically-grown *Acanthamoeba castellanii*
AC_b	*Acanthamoeba castellanii* grown on bacteria suspensions
APES	3-aminopropyltriethoxysilane
bp	base pair(s)
DAB	diaminobenzadine
DEPC	diethylpyrocarbonate
EGM 2%	endothelial growth medium supplemented with 2% FCS
EGM 20%	endothelial growth medium supplemented with 20% FCS
FCS	foetal calf serum
H&E	haematoxylin and eosin
H-EYE	*Hartmannella* sp isolated from corneal biopsy of patient GM
HEPES	N-2-hydroxyethylpiperazine N'-2-ethane sulfuric acid
ISH	*in situ* hybridisation
LEE	axenically grown strain of *Naegleria fowleri*
LEEmp	mouse passaged strain of *Naegleria fowleri*
PAS	periodic acid schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PYG	proteose peptone-yeast-glucose medium
(r)DNA	(ribosomal) deoxyribonucleic acid
RNA	ribonucleic acid
SB	Southern blotting

SEM	scanning electron microscopy
sp	species
TBS	Tris buffered saline
V-EYE	*Vahlkampfia* sp isolated from corneal biopsy of patient GM

SUMMARY

Acanthamoeba may cause a painful, potentially blinding, opportunistic infection of the cornea. The incidence of *Acanthamoeba* keratitis has increased in line with contact lens usage. Only in the last five years, however, has it been recognised that amoebae other than *Acanthamoeba* may also be associated with keratitis. Several reports implicating *Vahlkampfia* and *Hartmannella* now exist. These relatively unknown genera of free-living amoebae had not previously been recognised as causal agents of disease. This thesis investigated the pathogenicity of *Acanthamoeba* for corneal cells and tissue *in vitro,* compared, both quantitatively and qualitatively, to that of *Vahlkampfia* and *Hartmannella* isolates from a case of amoebal keratitis, termed V-EYE and H-EYE (Aitken *et al.*, 1995). Problems pertinent to the diagnosis of *Acanthamoeba*, *Vahlkampfia* and *Hartmannella* species (sp) in human corneal tissue were also addressed.

Identification of amoebae in pathological specimens is difficult. Indeed routine stains do not even adequately differentiate trophozoites from other cells. In this study calcofluor white similarly facilitated recognition of cysts but not trophozoites, while Grocott-Gomori's methenamine silver and Alcian blue critical electrolyte concentration techniques demonstrated both forms of amoebae only poorly.

Immunodiagnosis, however, with polyclonal antisera putatively specific for *Acanthamoeba*, facilitated recognition of both trophozoite and cyst forms. All 6 anti-amoebal antisera tested cross-reacted with all 3 genera of amoebae, as demonstrated by indirect immunofluorescence. Using SK anti-*Acanthamoeba* antiserum and the ABC-Peroxidase technique, different optimal dilutions of primary antibody were demonstrated for *Acanthamoeba, Vahlkampfia* and *Hartmannella* sp; run-to-run variation was unfortunately sufficiently marked to preclude differential diagnosis. Some background labelling of corneal tissue was observed with this antiserum, particularly associated with areas of tissue

disruption. Using the indirect alkaline phosphatase technique, however, no cross-reactivity was demonstrated for corneal or immune cells, even if pre-exposed to *Acanthamoeba castellanii.*

Polymerase chain reaction (PCR), using primers P1 and P2 (Lai *et al.*, 1994), was also capable of detecting both trophozoite and cyst forms of *Acanthamoeba castellanii.* The sensitivity of the standard 'cold' PCR method was 10 organisms or less. Its potential to detect amoebae other than *Acanthamoeba castellanii* is, however, uncertain The effect of altering PCR conditions to affect stringency was assessed. Despite using 'hot' PCR to detect one bp (base pair) differences in product, no set of conditions was found which made the method i) specific for *Acanthamoeba castellanii* only, ii) capable of detecting all *Acanthamoeba* sp tested but not members of the *Vahlkampfia* and *Hartmannella* genera, iii) capable of detecting representatives of all 3 genera. Run-to-run variation compounded the difficulties of interpreting the significance of a faint band on a gel. In spite of the usual precautions, contamination was problematic, limiting the potential of this technique for diagnostic use.

Following optimisation of a standard *in situ* hybridisation (ISH) method, the 126-bp probe complementary to the PCR product was successfully used to demonstrate *Acanthamoeba castellanii* in both cytospin preparations and corneal tissue. The method also facilitated detection of representatives of the 3 genera tested, except V-EYE. Within-run labelling, however, was inconsistent: poor penetration of intact organisms, particularly cysts, may have contributed, especially with cytospin preparations. Although at present ISH offers no advantages compared to immunocytochemistry, this feasibility study demonstrates the potential for differential diagnosis with genus-specific probes as gene sequence data becomes available.

With regard to pathogenicity, *Acanthamoeba*, *Vahlkampfia* and *Hartmannella*

each successfully produced stromal invasion when incubated with eyebank corneas for 24 hours. Similar preliminary studies with rabbit corneas were unsuccessful. Studies on human corneas inoculated with *Acanthamoeba castellanii* demonstrated migration throughout the stroma within 24 hr, encystation in line with keratocyte depletion and little associated stromal disruption. This agrees with other evidence suggesting that much of the pathology observed in clinical specimens is due to the immune response, although deleterious effects of therapy may also contribute.

Cultured keratocytes exposed to *Acanthamoeba castellanii* demonstrated marked destruction of keratocytes (>75% of the 'monolayer') within 24 hr at a concentration of 10^6/ml/well. No cytopathic effect was demonstrated with trophozoite concentrations below 10^4/ml/well. At all concentrations tested rounding-up/encystation occurred with time, despite residual keratocytes. Comparing the 3 genera (10^6/ml/well), although a greater cytopathic effect was demonstrated for axenically cultured *Acanthamoeba castellanii* after only 6 hr of incubation, monoxenically-cultured V-EYE and H-EYE also produced near complete destruction of keratocytes by 24 hr.

Cultured epithelial cells were shown to be less susceptible than keratocytes to the cytopathic effect produced by *Acanthamoeba castellanii*. The increased resistance was mainly due to a slower initial rate of destruction. The tight junctions of the epithelial monolayer contrast with the haphazard growth pattern of the stromal keratocytes: it is postulated that the former serve as a relatively effective barrier against amoeba. Indeed, invasion was demonstrated with human eyebank corneas, where the epithelium is degenerate, but not with fresh rabbit corneas. This theory concurs with the requirement, demonstrated in other studies, for direct inoculation or abrasion of the corneal surface in animal models. This in turn supports the widely accepted belief that penetration of the cornea requires some degree of compromise of the epithelium, hence the marked association with contact lens wear.

Cell culture studies investigating the mechanisms of cell destruction revealed that amoebae physically insinuate themselves between adjacent cells and between cells and the substratum, thus mechanically weakening their contacts. The resulting retraction fibres were particularly vulnerable. The role of the *Acanthamoeba castellanii* finger-like processes, observed forming contact with target cells, is unknown. Cells were destroyed by piece-meal phagocytosis (i.e. trogocytosis). The intracellular contents of trophozoites, however, also produced cell destruction, albeit more slowly, as did secretions produced by amoebae co-incubated, but not in physical contact, with keratocytes. Cell damage in these instances was manifest as increased granularity and vacuolisation with rounding up, detachment and cell death. A role for apoptosis in this process was not demonstrated for *Acanthamoeba castellanii*. All the observations listed in this paragraph, except where *Acanthamoeba castellanii* is specified, refer also to V-EYE and H-EYE. The results suggest that, not only are V-EYE and H-EYE as cytopathic as *Acanthamoeba castellanii*, but also that the mechanisms of cell destruction involved are similar for the 3 genera.

TABLE 1.3

Acanthamoeba Species Classification - Pussard and Pons

Group I	Group II	Group III
A astronyxis	*A castellanii*	*A palastinensis*
A comandaii	*A mauritaniensis*	*A culbertsonii*
A echinulata	*A polyphaga*	*A leuticulata*
	A lugdenensis	*A pustulosa*
	A quina	*A royreba*
	A rhysodes	
	A divionensis	
	A paradivionensis	
	A griffini	
	A triangularis	

TABLE 1.1

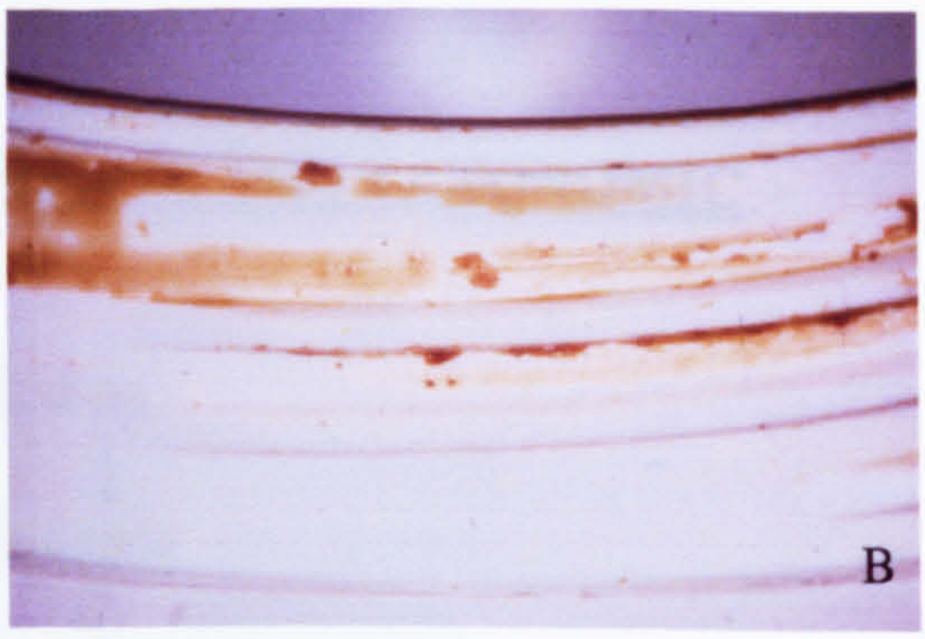
THE POSITION OF *ACANTHAMOEBA* IN THE TAXONOMIC SCHEME

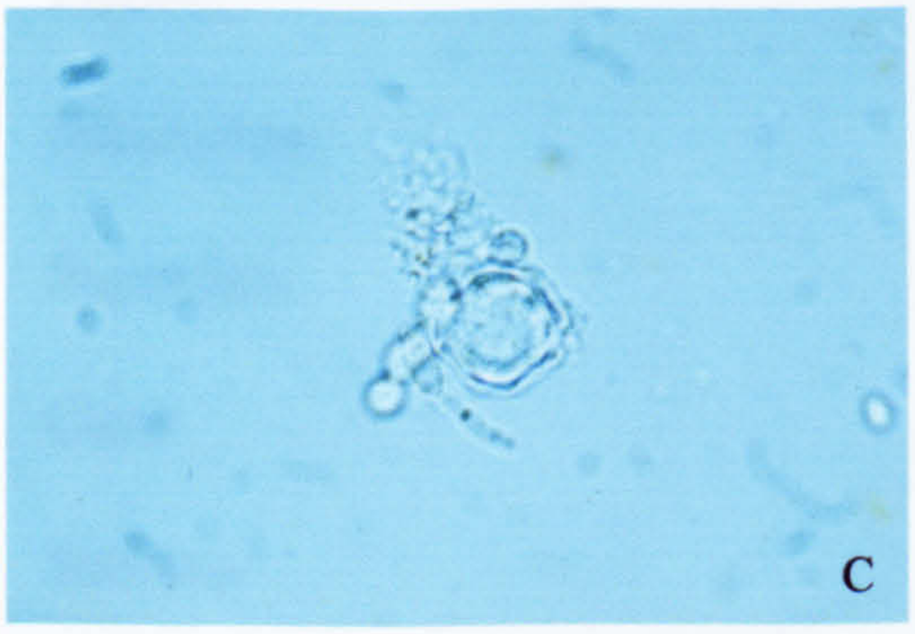
Kingdom	Protista		
Subkingdom	Protozoa		
Phylum	Sarcomastigophora		
Subphylum	Sarcodina		
Superclass	Rhizopodea		
Class	Lobosea		
Subclass	Gymnamoebia		
Order	Amoebida	Amoebida	Schizopyrenida
Suborder	Tubulina	Acanthopodina	
Family	Hartmannellidae	Acanthamoebidae	Vahlkampfiidae
Genus	*Hartmannella*	*Acanthamoeba*	*Vahlkampfia*

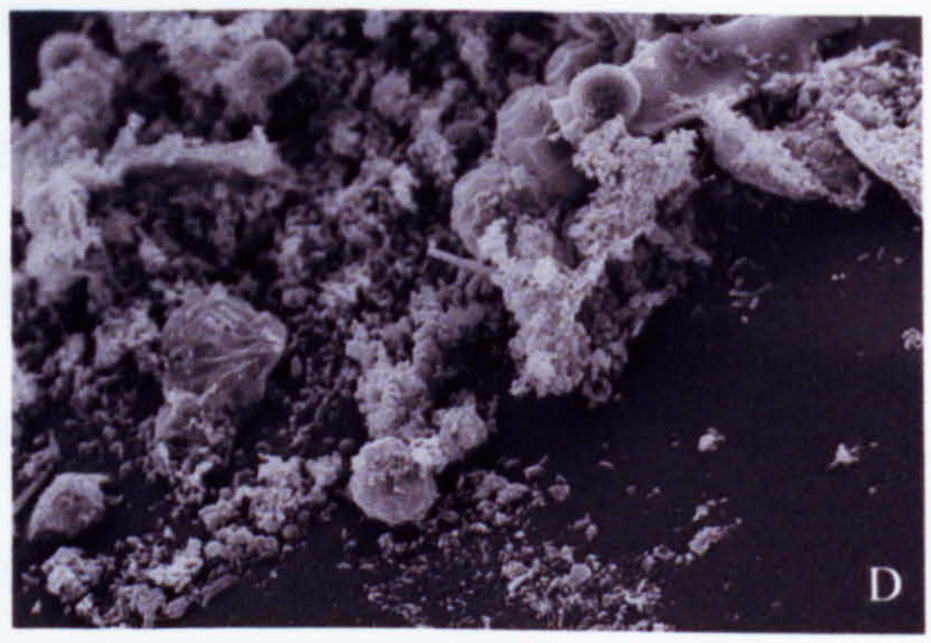
TABLE 1.2

REVISED POSITION OF *ACANTHAMOEBA* IN THE TAXONOMIC SCHEME

Class	Lobosea		Heterolobosea
Subclass	Gymnamoebia		
Order	Euamibida	Acanthopodida	Schizopyrenida
Family	Hartmannellidae	Acanthamoebidae	Vahlkampfiidae
Genus	*Hartmannella*	*Acanthamoeba*	*Vahlkampfia*







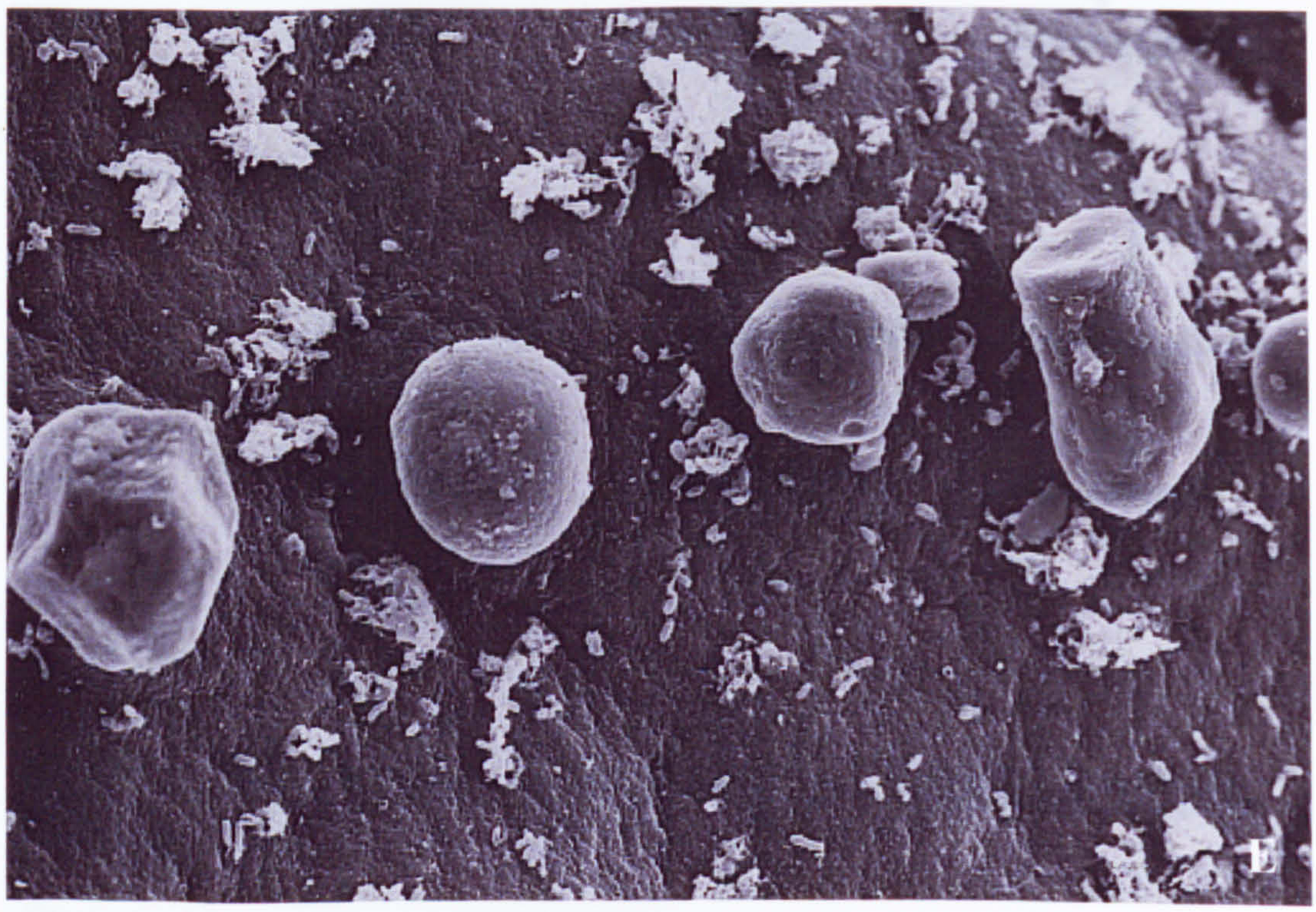


Fig 1.6 Contact lens contaminants

A) Contact lens storage case system.
B) Debris around grooves of screw-on mechanism of lid of contact lens storage case.
C) Phase contrast micrograph of contaminants derived from contact lens storage case comprising of amoebal cyst, bacteria and fungal elements. (x340)
D) Scanning electron micrograph of amoebal cysts and bacteria in contact lens biofilm. (x800)
E) Scanning electron micrograph of contact lens surface demonstrating bacteria and a row of amoebae. The amoebae appear to be neither true cysts nor trophozoites but are of an intermediate form. (x2800)

(A, B & C- courtesy of Dr J Hay; D & E- courtesy of D Aitken)

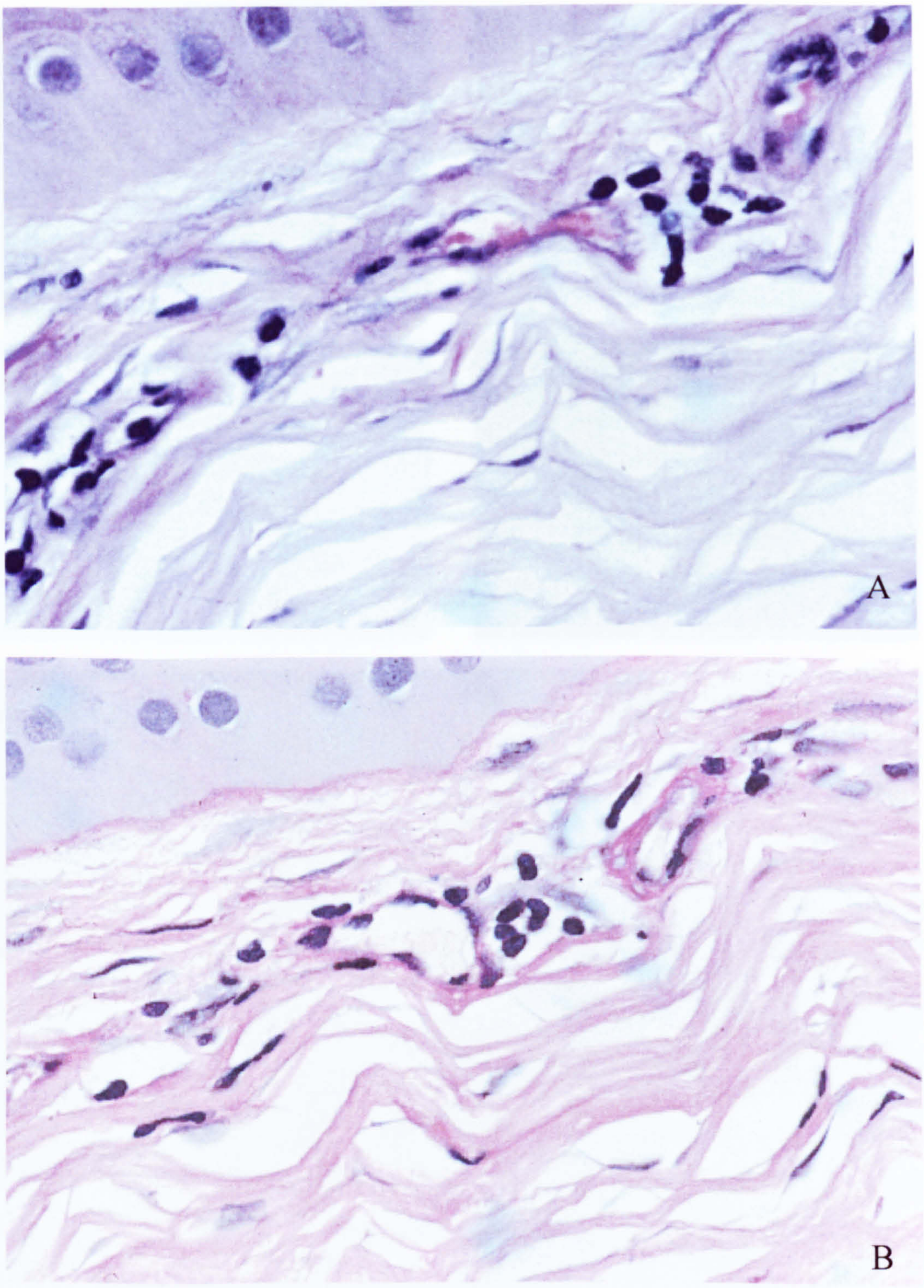


Fig 1.5 Demonstration of amoebal trophozoites by nonspecific histological stains

A) Giemsa-stained section of corneal biopsy specimen from patient GM (Aitken *et al*, 1995).
B) PAS-stained preparation of same specimen.

The stroma contains keratocytes and blood vessels surrounded by inflammatory cells. Although amoebal pathogens were isolated on culture and the presence of the organisms was demonstrated with anti-*Acanthamoeba* antiserum (see Fig 1.3), trophozoites can not be identified with any certainty in these sections, nor with H&E staining.

(Light microscopy: mag x300 - Courtesy of Professor WR Lee)

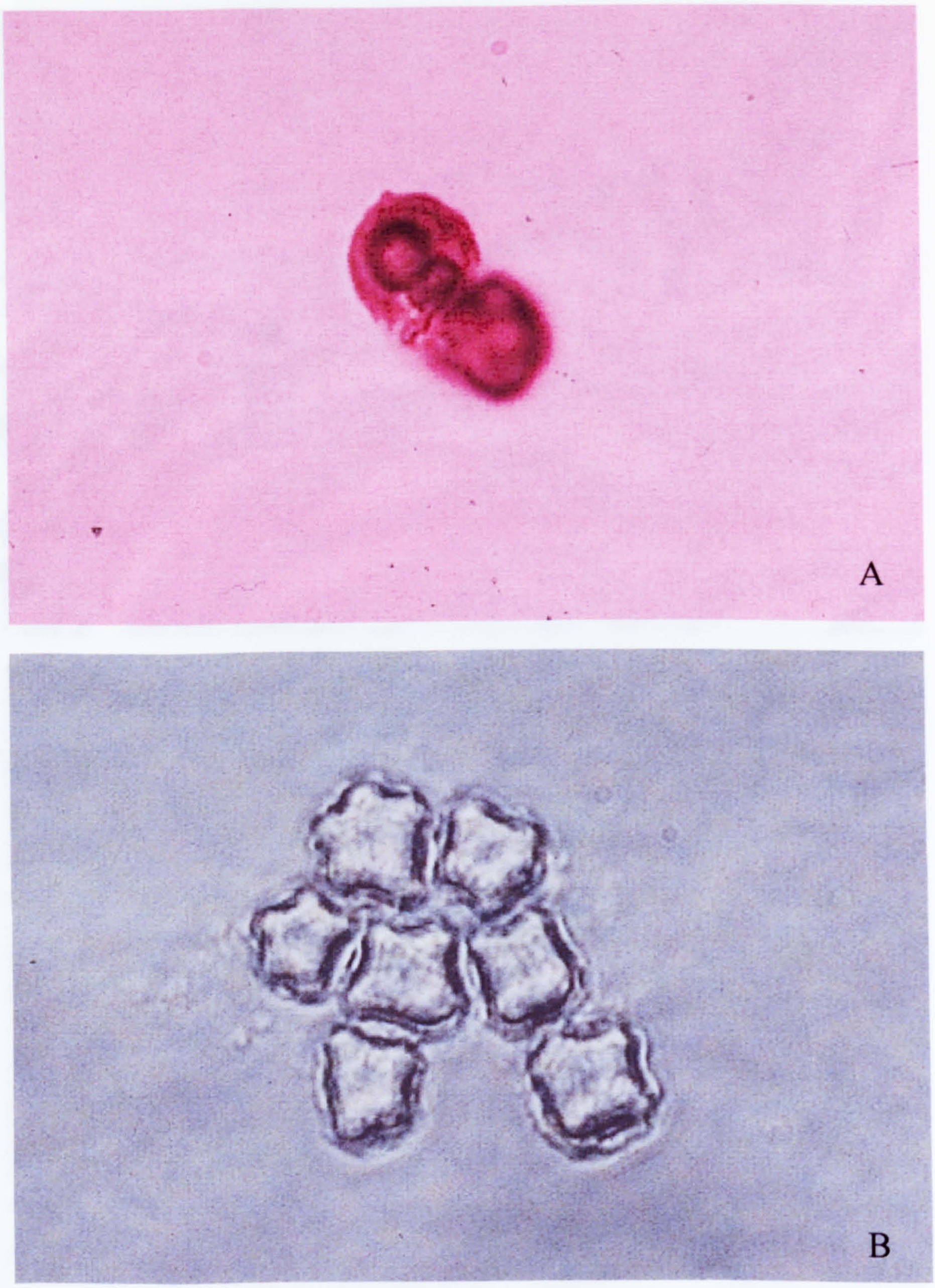
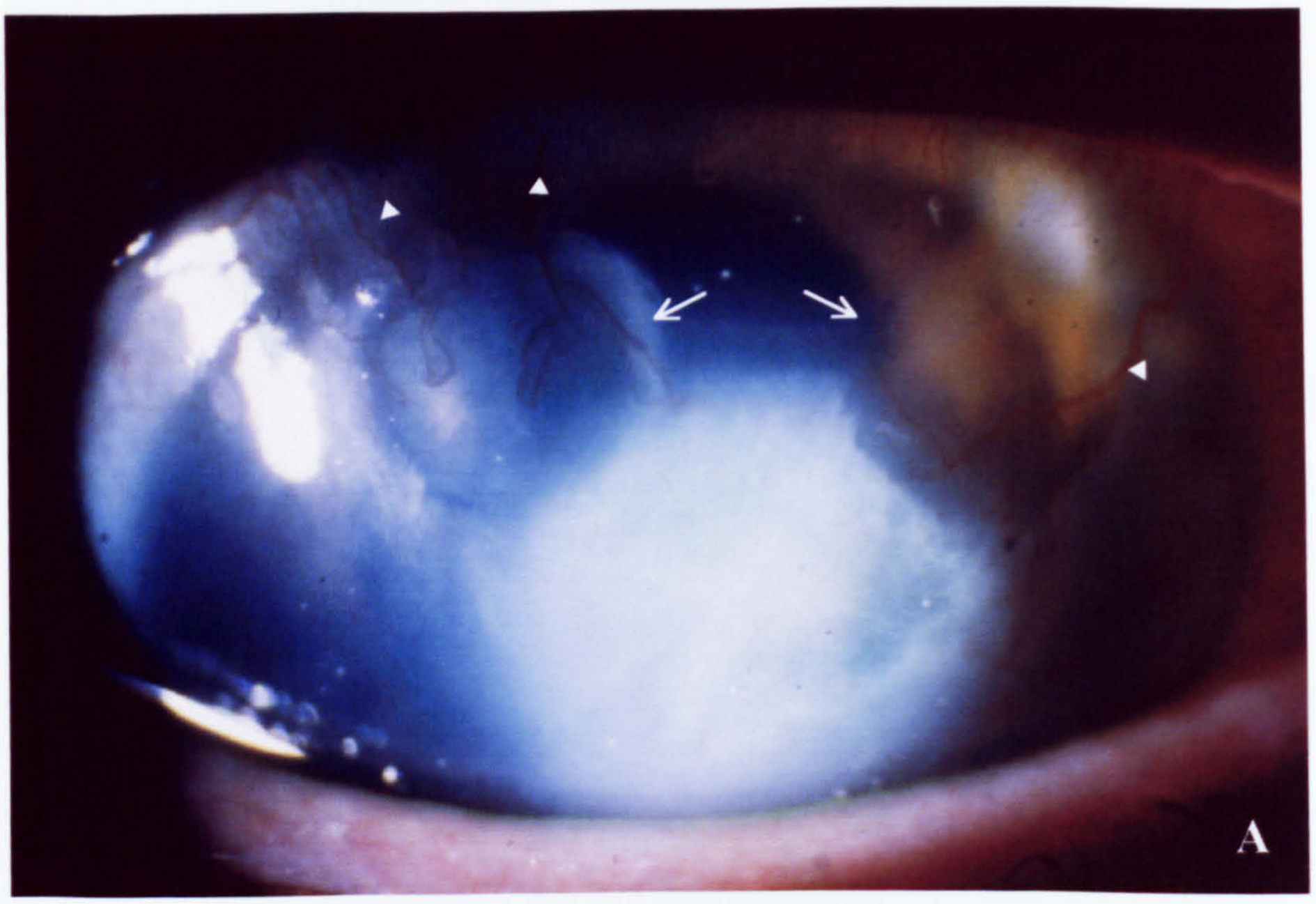


Fig 1.4 Amoebal cysts

A) Appearance of degenerate amoebal cysts in direct wet mount examination of fluid in which biospy specimen was transported.

B) Cultured cysts of *Acanthamoeba castellanii*, as viewed on the same microscope, for comparison.

(Phase contrast microscopy: mag x680)



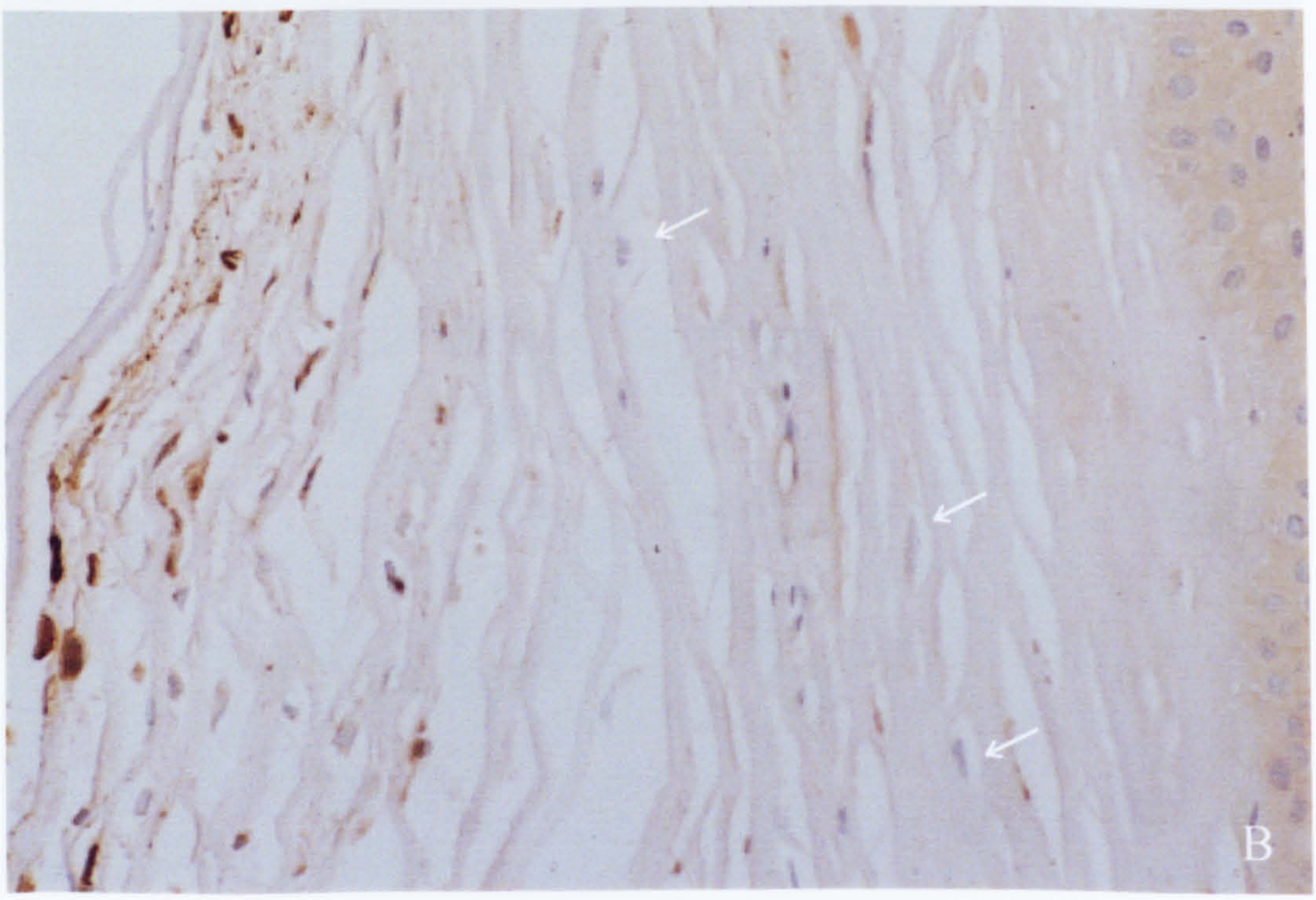
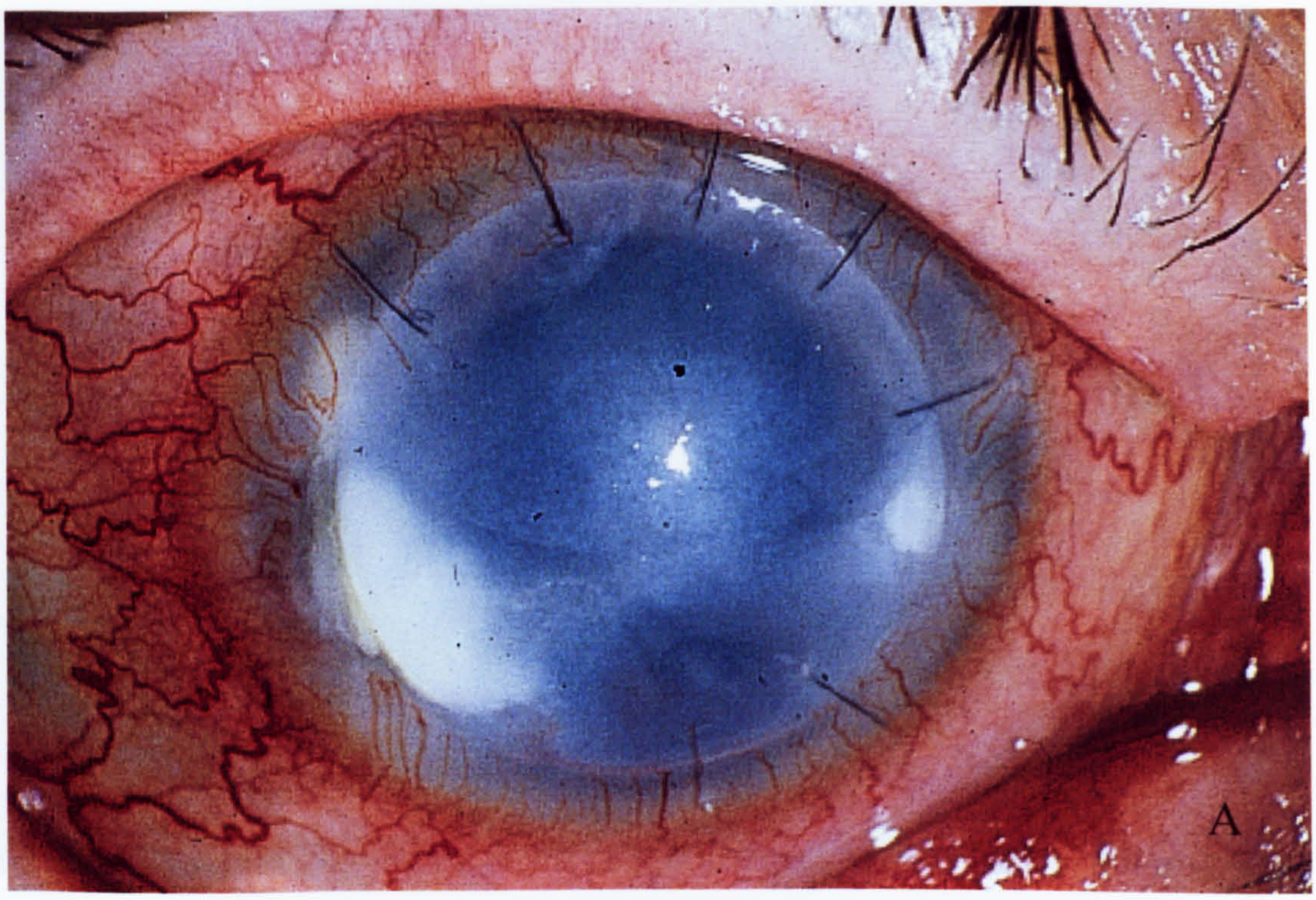


Fig 1.3 Non-*Acanthamoeba* amoebal keratitis

A) Advanced infection in patient GM with non-*Acanthamoeba* amoebal keratitis (Aitken *et al*, 1995). Note stromal biopsy sites (arrows) and also the presence of vascularisation (arrowheads). The latter is not normally observed even in advanced cases of *Acanthamoeba* keratitis. (Courtesy of Professor CM Kirkness)

B) Immunoperoxidase-staining of a section from the biopsy specimen of patient GM utilising an anti-*Acanthamoeba* polyclonal antiserum. Note that labelled trophozoites can now be more readily distinguished from the morphologically similar keratocytes (arrows). (Light microscopy: mag x120 - Courtesy of Professor WR Lee)



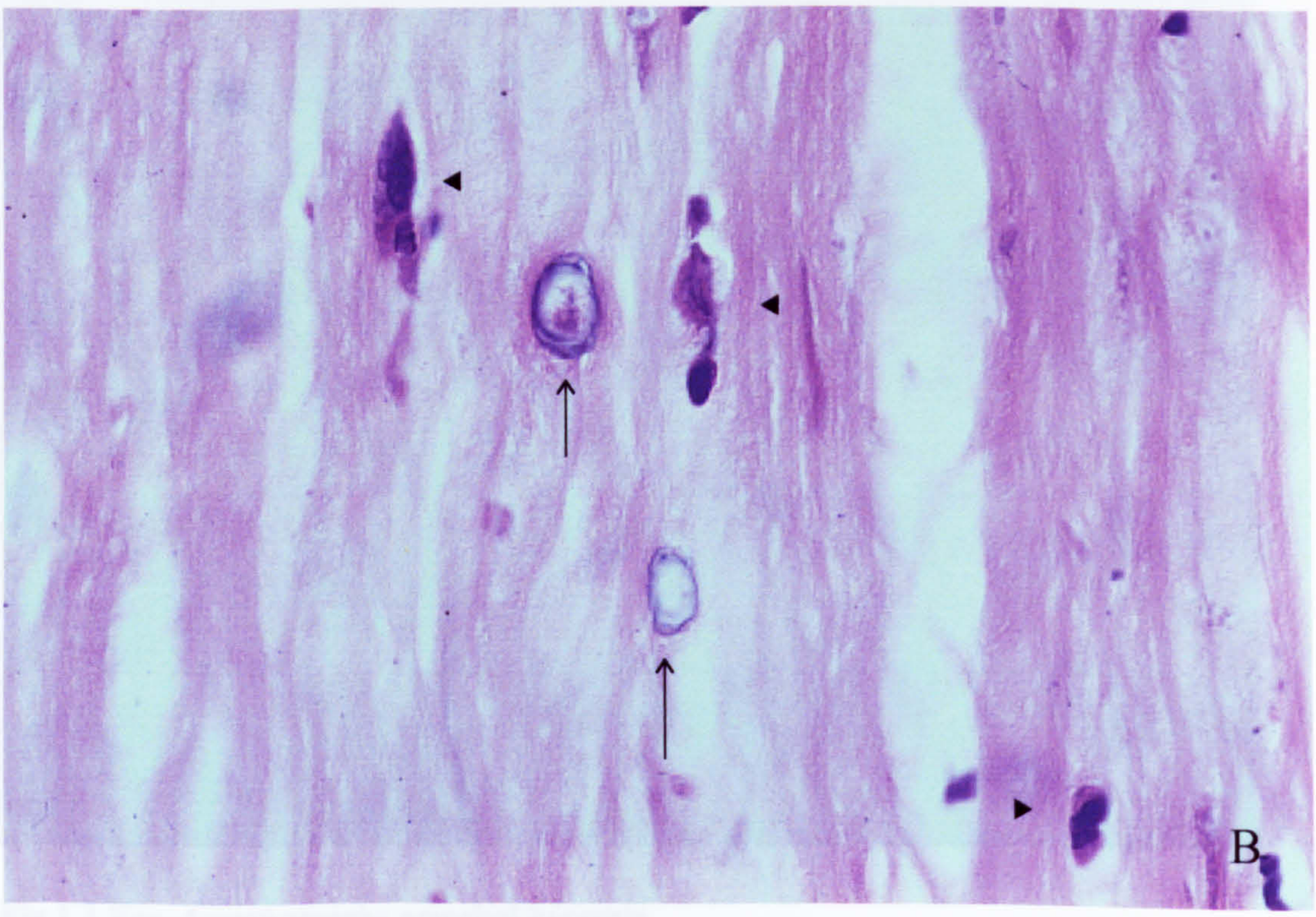
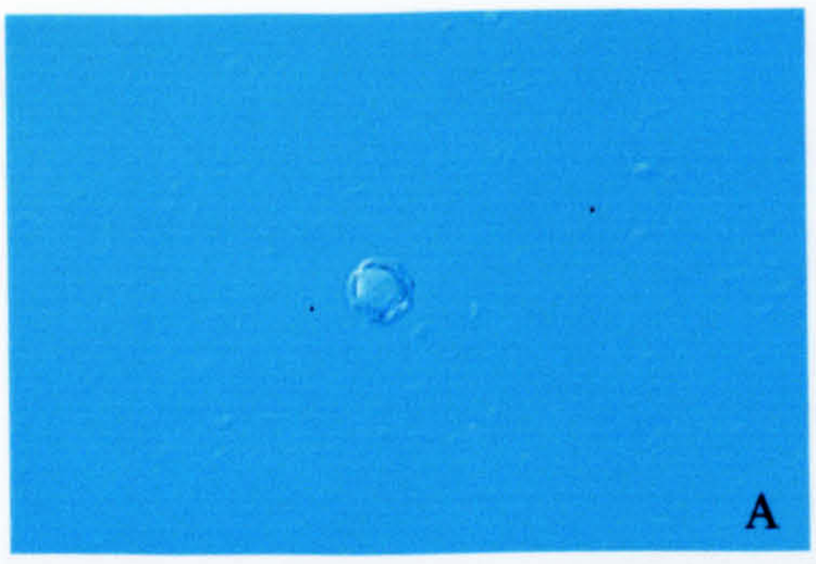
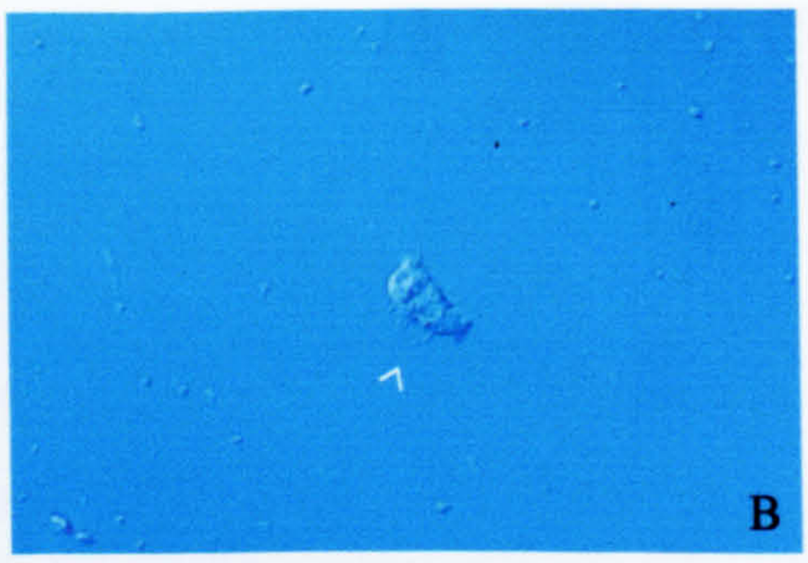


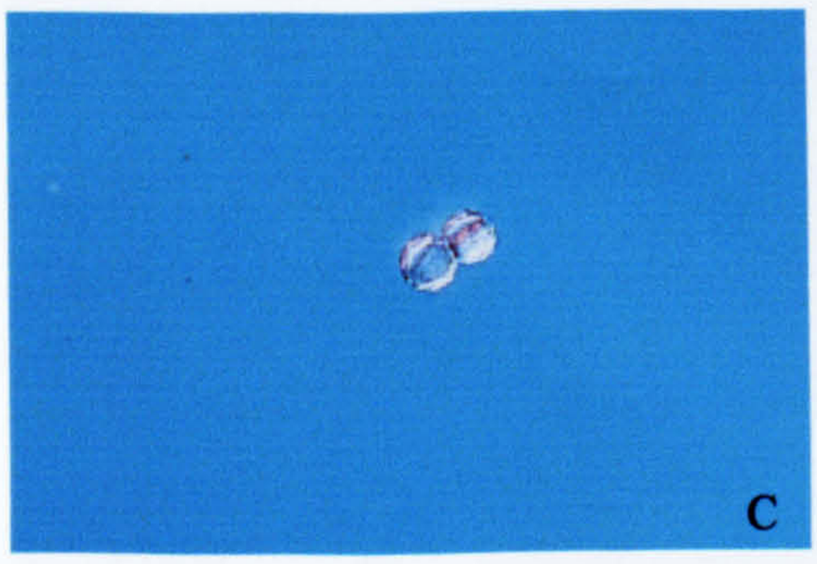
Fig 1.2 *Acanthamoeba* keratitis

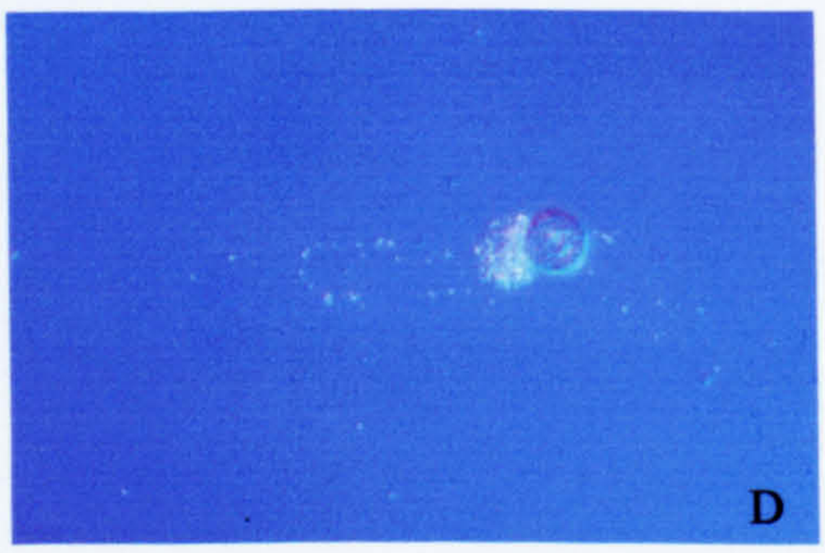
A) Inflamed eye with corneal opacity associated with recurrence of *Acanthamoeba* keratitis in a corneal graft. (Courtesy of Professor CM Kirkness)

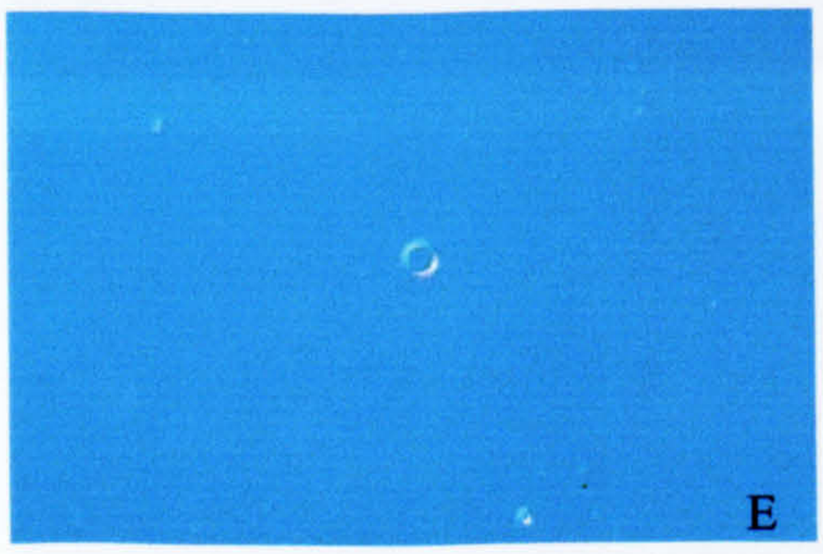
B) PAS-stained biopsy preparation illustrating amoebae infiltrating between the stromal lamellae. Cysts (arrows) are readily identified but trophozoites may be difficult to differentiate from keratocytes (see arrowheads). (Light microscopy x300 - Courtesy of Professor WR Lee)

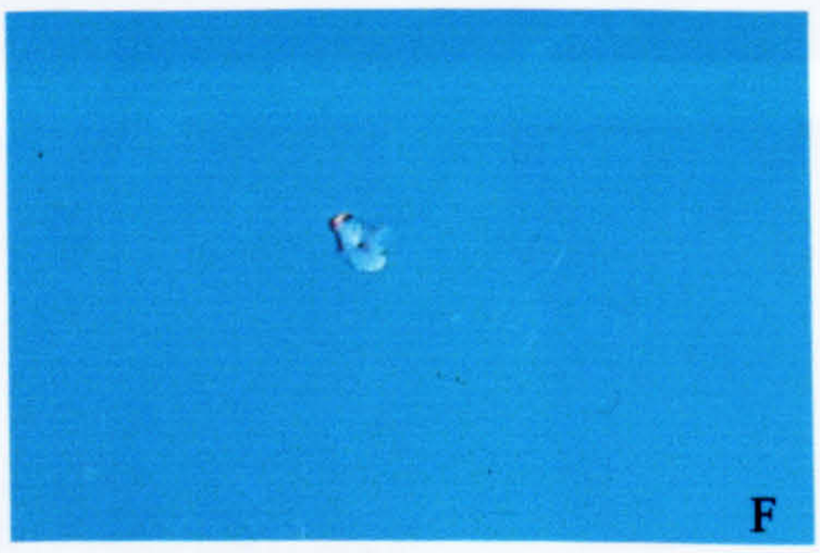


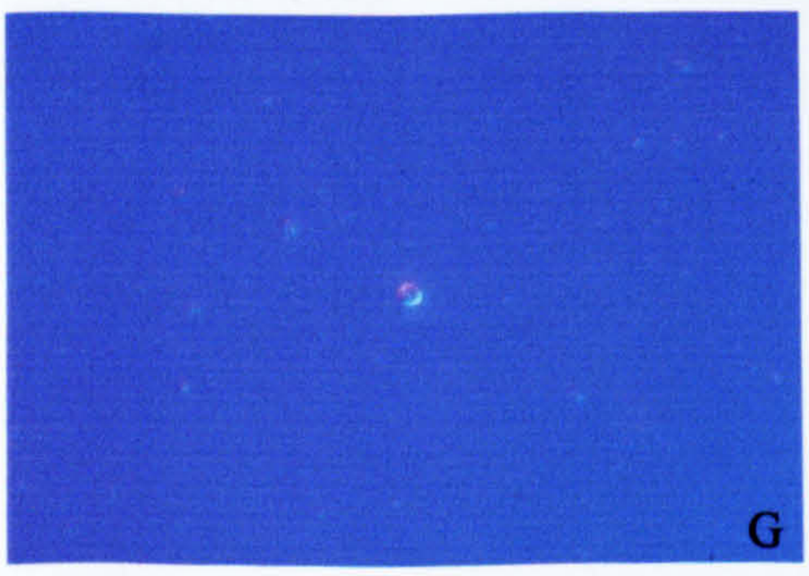












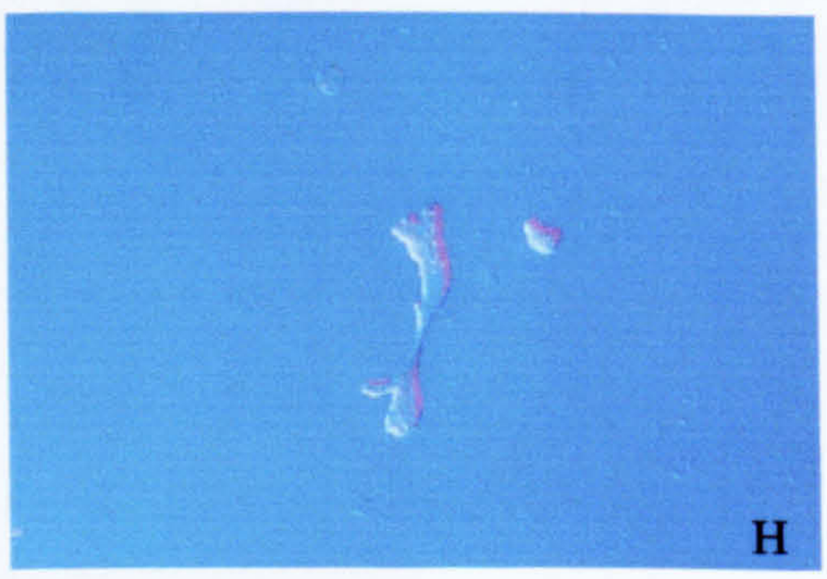


Fig 1.1 Amoebal cysts and trophozoites:

A) *Acanthamoeba castellanii* cyst B) *Acanthamoeba castellanii* trophozoite
C) *Acanthamoeba polyphaga* cyst D) *Acanthamoeba polyphaga* trophozoite
E) *Vahlkampfia* cysts (V-EYE) F) *Vahlkampfia avara* trophozoite
G) *Hartmannella* cysts (H-EYE) H) *Hartmannella vermiformis* trophozoites

H-EYE cysts are not illustrated but are very similar to those of *Hartmannella vermiformis. Vahlkampfia avara* cysts are also similar to those of V-EYE except that they lack the surrounding sticky coat apparent with V-EYE (see Fig 2.1). Note the spiny acanthopodia typical of *Acanthamoeba* (B-arrowhead) and the trail of egested debris commonly found in the wake of amoebal trophozoites (D).

(Interference contrast: mag x170)

1. LITERATURE REVIEW

1.1 General Overview

1.1.1 Introduction

Acanthamoeba is a free-living amoeba that feeds on other environmental micro-organisms. It exhibits both cyst and trophozoite forms (Fig 1.1). Relevant details concerning the biology of this fascinating protozoan are elaborated upon in Section 1.1.3. The organism has a worldwide distribution and is ubiquitous in the environment (Rodriguez-Zaragoza, 1994). It can be isolated from air, soil and both fresh and salt water, including swimming pool and tap water.

Members of the genus were first recognised in cultures of soil prepared for the isolation of bacteria and fungi (Castellani, 1930). It was initially believed that all known species were harmless. The ability to produce a cytopathic effect was not reported until 1957 (Jahnes *et al.*, 1957). The following year Culbertson and colleagues (1958 &1959) described a species of *Acanthamoeba* capable of killing experimental animals. It is now well recognised that *Acanthamoeba* is capable of opportunistic pathogenicity in animals, including humans.

Neural infection in humans causes a slowly progressive chronic granulomatous encephalitis (Martinez, 1991). This is a rare disease, which usually occurs in immunocompromised patients: it is sometimes associated with disseminated disease (Sison *et al.*, 1995). The route of invasion is assumed to be haematogenous, probably from a primary focus in the respiratory tract or through ulceration of the skin (Martinez, 1983; Ma *et al.*, 1990; Martinez, 1991; John, 1993).

Acanthamoeba may also cause localised infections. Apart from pneumonitis

and skin lesions, there are single reports of infection of the ear (Jakovljevich *et al.*, 1969) and a mandibular bone graft (Borochovitz *et al.*, 1981). Most commonly, however, *Acanthamoeba* invades the cornea, producing *Acanthamoeba* keratitis (Fig 1.2).

Acanthamoeba keratitis is a potentially blinding infection that can be exceedingly painful. The condition has been recognised only relatively recently, the first reported cases occurring in both the USA and the UK circa 1973 (Nagington *et al.*, 1974; Jones *et al.*, 1975a; Jones *et al.*, 1975b). It is generally assumed that, due to lack of clinical suspicion and difficulties in detection, some earlier cases may have been unrecognised. Evidence of any cases prior to 1973 is, however, lacking (Kelly, 1992) although this may be partly due to lack of archival specimens (Kirkness *et al.*, 1994).

Historically the condition was associated with corneal trauma ((Nagington *et al.*, 1974; Jones *et al.*, 1975a; Jones *et al.*, 1975b; Auran *et al.*, 1987) and indeed such cases still occur (Sharma *et al.*, 1990). The incidence of *Acanthamoeba* keratitis has, however, increased markedly in modern industrial societies since the 1980s in line with increasing contact lens wear, which is now the biggest single risk factor (Moore *et al.*, 1987; Bacon *et al.*, 1993b; Schaumberg *et al.*, 1998). Estimates of incidence vary (Schaumberg *et al.*, 1998), but a prospective culture-proven cohort study in the population of the West of Scotland in 1994 and 1995 has estimated the incidence of *Acanthamoeba* keratitis at 1:6750 contact lens wearers per year (Dr D.V. Seal - personal communication). It is largely a disease of young, healthy individuals whose vision was previously normal when corrected with respect to refractive error. If not diagnosed in the early stages, prolonged medical therapy and repeat surgical procedures may be required (Section 1.1.4). The resulting effects on both vision and quality of life may be severe. It is also of note that contact lens wear is becoming increasingly commonplace in less developed

countries where, not only is the climate often hot and dusty, but patient education, medical support and hygiene conditions may all be suboptimal.

Hence the ability of members of the free-living *Acanthamoeba* genus to produce opportunistic infection is of some importance from a medical point of view. Various studies on contact lens usage have been performed to attempt to identify risk factors associated with development of *Acanthamoeba* keratitis (Radford *et al.*, 1995; Illingworth *et al.*, 1995). An understanding of the pathogenesis of the condition is crucial for effective reduction of the incidence of *Acanthamoeba* keratitis. The pivotal role of the contact lens in facilitating invasion of the immunopriviledged cornea is discussed in Section 1.3.4.

Pathogenicity, or the ability to produce disease, is the property of a species and thus a taxonomically significant attribute. Virulence refers to the relative pathogenicity of a particular strain. The factors that contribute to virulence are discussed in Section 1.3.5.

Of the approximately 17-20 named species of *Acanthamoeba*, 7 have so far been associated with keratitis. *Acanthamoeba castellanii, polyphaga* and *culbertsoni* are the most common causal agent (Kirkness *et al.*, 1994) but *hatchetti* (Cohen *et al.*, 1987), *rhysodes* (John, 1993), *lugdunensis* (Matias, 1991), and *griffinii* (Ledee *et al.*, 1996) have also been described. Speciation of *Acanthamoeba* is, however, a contentious subject and is currently under review (Section 1.1.2).

It has been suggested that only a subset of *Acanthamoeba* is capable of

producing keratitis (De Jonckheere, 1991). A preliminary study using a rat model (Badenoch *et al.*, 1990) also suggests that only particular subsets of *Acanthamoeba* are virulent in the human cornea. At present, however, it appears that a single species in the genus can comprise a mixture of pathogenic and non-pathogenic strains (Bogler *et al.*, 1983). There may therefore be a range of pathogenic potential among these essentially opportunistic agents, rather than a sharp cut off point between those that are capable of causing disease and others that are not. Indeed, two publications have suggested that virulence may not be a constant feature of a particular strain (Stevens & O' Dell, 1974; Cursons & Brown, 1978) and it has even been suggested that all *Acanthamoeba* may be potentially pathogenic (John, 1993).

Two other genera of free-living amoebae, *Vahlkampfia* and *Hartmannella,* have also recently been implicated in corneal infection (Aitken *et al.*, 1996). The *Vahlkampfia* and *Hartmannella* sp used in this study were originally isolated from a corneal biopsy from this particular patient (GM) and are termed V-EYE and H-EYE (Fig 1.1 & 1.3). In several other cases evidence has been obtained to suggest that amoebae other than *Acanthamoebae* sp may be associated with keratitis (Seal *et al.*, 1996; Kennedy *et al.*, 1995; Kinnear *et al.*, 1995; Alexandrakis *et al.*, 1998; Aimard et al., 1998; Inoue et al., 1998).

Even for an experienced protozoologist, differentiating these organisms in pathology specimens on the basis of morphology may be difficult (Section 1.2.3). Furthermore, as *Vahlkampfia* and *Hartmannella* had not previously been implicated in keratitis, the potential cross-reactivity of existing diagnostic methods for *Acanthamoeba* had not been investigated. It is therefore theoretically possible that previous cases may have been overlooked, or alternatively misdiagnosed as *Acanthamoeba* keratitis. Drug sensitivity screening *in vitro* suggests that therapies other than those classically used to treat *Acanthamoeba* keratitis may be more effective in cases of keratitis

associated with these other genera of amoebae (Aitken *et al.*, 1996). There is thus a need for assessment of existing diagnostic methods with respect to cross-reactivity, and for development of new methods, both for pan-amoebae and differential diagnosis (Section 1.2 and Chapt 3).

With regard to recognition of novel amoebal pathogens, it is of interest that it was only in 1990 that *Balamuthia mandrillaris* was recognised as a causative agent of meningitis (Visvesvara *et al.*, 1990; Visvesvara *et al.*, 1993). The potential for *Vahlkampfia* and *Hartmannella* to be pathogenic in humans is, however, controversial (De Jonckheere + Brown, 1998 a, b, c). Two cases of encephalitis putatively due to *Vahlkampfia* have been reported; the diagnosis was based purely on the morphological appearance of the amoebae in tissue sections (De Jonckheere, 1987). A fatal case of meningoencephalitis has been reported in association with *Hartmannella vermiformis*; gram positive cocci were, however, also isolated (Centeno *et al.*, 1996).

In patient GM, *Acanthamoeba*, *Vahlkampfia* and *Hartmannella* were all present in the contact lens case, but only *Vahlkampfia* and *Hartmannella* were cultured from the corneal biopsy specimen (Aitken *et al.*, 1996). These organisms were isolated from a scrape of the base of the stromal biopsy site, with the protozoologist in attendance. It is therefore considered unlikely that they were contaminants or transients. To what extent the *Vahlkampfia* and *Hartmannella* were contributing to the observed tissue damage is unknown, as is whether or not they are capable of initiating infection in isolation. It is therefore important to confirm the hypothesis that these organisms are pathogenic in corneal tissue and to compare their potential to produce cell damage, both quantitatively and qualitatively, with that of *Acanthamoeba* (Section 1.3 and Chapt 4).

Throughout this thesis the term *Acanthamoeba* keratitis is used to refer only to corneal infection with members of this specific genus. In contrast, amoebal

keratitis is defined as corneal infection with any of the 3 genera *Acanthamoeba*, *Vahlkampfia* and *Hartmannella*.

1.1.2 Taxonomy

The taxonomy of small free-living amoebae is the subject of ongoing discussion in the literature. It should be noted that the first member of the genus to be recognised, the amoeba now known as *Acanthamoeba castellanii*, was originally termed *Hartmannella castellanii* (Douglas, 1930) following its isolation from a yeast-like fungal culture (Castellani, 1930). The genus *Hartmannella* was subsequently redefined as 3 separate genera, creating *Acanthamoeba* as a new genus (Volkonsky, 1931). Much confusion has surrounded its nomenclature and taxonomy, which has been revised many times (Visvesvara, 1991). The redefinition by Page (1967b) which followed the study of a range of free-living amoebae (Page, 1967a & 1967b) basically underpins the accepted modern classification of *Acanthamoeba, Vahlkampfia* and *Hartmannella* (Page, 1988). This is outlined below and is based on morphological differences in trophozoites and cysts, nuclear division and locomotion.

The position of *Acanthamoeba* in the taxonomic scheme of the Society of Protozoologists (Bovee, 1985) is shown in Table 1.1. This has since been updated by Page (Page, 1988). In this classification (Table 1.2) *Acanthamoeba, Vahlkampfia* and *Hartmannella* are now recognised not just as different genera of amoebae: they are separated at the order level in the case of *Acanthamoeba* and *Hartmannella* and at the class level with regard to *Vahlkampfia*. *Vahlkampfia* is therefore more closely related to *Naegleria*, a genus of the Vahlkampfiidae family which is known to cause an acute meningoencephalitis in immunocompetent individuals, than to *Acanthamoeba* and *Hartmannella*.

Mitochondrial DNA studies have indeed demonstrated genetic similarity between amoebae from the genus *Vahlkampfia* and strains of *Naegleria gruberi*

(Milligan & Band, 1988). Phylogenetic studies based on 18S (Sogin, 1989) and 5S (MacKay & Doolittle, 1981) ribosomal RNA sequences suggest that *Acanthamoeba* is at least as closely related to higher animals and plants as to other protozoa. Other studies on small subunit RNA have also shown that the lobose amoebae are polyphyletic in origin with *Acanthamoeba* and *Naegleria* having separate origins (Clark & Cross, 1988, Baverstock *et al.*, 1989). Work has concentrated on these 2 genera due to their known involvement in human disease.

Thus taxonomists generally agree that distinctions among amoebae are relatively clear at the genus level; as with other asexual organisms, however, the concept of species is much less clear. Speciation of *Acanthamoebae* by morphology alone is difficult because it is based on much more subtle differences. Furthermore morphological features may vary with culture conditions (Sawyer, 1971; Stratford & Griffiths, 1978).

Attempts to clarify the classification of *Acanthamoeba* by including other features such as isoenzyme profiles and restriction fragment length polymorphisms have demonstrated discrepancies with traditional species boundaries (Visvesvara, 1991). For example, analysis of restriction length polymorphisms of mitochondrial DNA has also revealed a large degree of interstrain genetic diversity, suggesting polyphyletic origins (Bogler *et al.*, 1983; Yagito & Endo, 1990). Conversely certain strains of *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* give identical banding patterns (Bogler *et al*, 1983; Yagito & Endo, 1990; Costas *et al.*, 1983). Classification of species within the genus *Acanthamoeba* is therefore, at present, to be considered unreliable. Similar molecular studies of *Vahlkampfia* and *Naegleria* suggest that, while the species boundaries are more distinct, they are not completely resolved (Milligan & Band, 1988; Baverstock *et al.*, 1989; Brown & De Jonckheere, 1994). No such data are available for *Hartmannella*.

Hence restriction fragment length polymorphisms have highlighted ambiguities in the morphology-based classification. This is important because morphologically similar species may be highly divergent at the genomic level. This may be associated with differences in biochemistry and physiology with consequent effects on potential pathogenicity (see Section 1.3.5). Indeed it has been suggested that restriction fragment length polymorphisms may be useful in identifying potentially pathogenic strains (Kilvington *et al.*, 1991).

Classification of *Acanthamoeba* at the subgenus level is thus currently under review. Pussard and Pons (Pussard & Pons, 1977) have subdivided the genus *Acanthamoeba* into 3 morphological groups on the basis of cyst size and shape (Table 1.3). Group I have large cysts with a rounded outer cell wall (ectocyst) clearly separated from the inner wall (endocyst), to which it is joined by radiations that form a star-shaped structure. Group II cysts are smaller with a variable endocyst morphology and group III cysts are smaller again with poorly separated ecto- and endocyst walls. Page (1988) accepted this subclassification with a few changes. In a study designed to investigate the phylogeny of *Acanthamoeba*, partial sequencing of small subunit ribosomal RNA produced data concordant with this classification (Johnson *et al.*, 1990), although only 7 strains representing 5 species were involved.

This has been further investigated with particular reference to Pussard & Pons groups II & III. A preliminary study of 18 isolates of *Acanthamoeba* revealed that 15 of those isolates formed a tight phylogenetic cluster termed sequence type T4 (Gast *et al.*, 1996). The sequences of the remaining 3 isolates were each very different and were therefore designated sequence types T1-3. In a more detailed study from the same laboratory, involving 53 strains that included the original 18 isolates, it was demonstrated that each of the morphological groups consists of several distinct lineages identified as different sequence types (Stothard *et al.*, 1998). Correlations between the morphological groups, species and the sequence types revealed only 4 isolates that were discordant. The sequence types and species were not, however,

uniformly equivalent ie isolates with the same sequence type are currently classified as different species. It was therefore suggested that the 4 discordant isolates are in fact misclassified.

Gast and colleagues (1996) selected Pussard & Pons groups II & III for study because they are most commonly associated with human disease. Indeed all cases of keratitis so far reported have been associated with these 2 morphological groups (John, 1993; Matias *et al.,* 1991; Ledee *et al.*, 1996). In the more detailed study, Stothard and co-workers (1998) note that 17 of the 18 human keratitis isolates had T4 sequence types while *Acanthamoeba griffini* (Ledee *et al.*, 1996) is T3. Another 7 keratitis isolates, excluded due to incomplete sequence data, also belong to T4. They postulate that, as T3 and T4 diverge from a common ancestor, then the ability to cause keratitis may have evolved only once. It is, however, also possible that keratitis simply appears to be associated with T4 as they are prevalent in the environment. The data presented indicate that experimental and human neural infections have indeed been associated with other sequence types. This may also be found to be the case with keratitis as numbers of cases accumulate.

In summary, recent more detailed analysis of free-living amoebae, and in particular emerging molecular information, is not always consistent with older, morphological classifications. Consequently the concept of pathogenic and non-pathogenic species is not valid, at least until the species boundaries are more firmly established. It does, however, confirm that the three genera are markedly different at the genetic level. Hence classification of *Acanthamoeba, Vahlkampfia* and *Hartmannella* as 3 separate genera is not only justified but also important, as the biology and physiology of these organisms may be much more different than their morphological similarities would suggest.

1.1.3 Biology of *Acanthamoeba, Vahlkampfia and Hartmannella*

The life cycle of *Acanthamoeba* is simple, consisting of only two distinct

states, the trophozoite and the cyst, although intermediate forms are also described (John, 1993; Ma, 1990). The former is the feeding, motile, replicating stage. Encystation, and relative dormancy, occurs when the environment is unfavourable.

The trophozoite form of *Acanthamoeba* is characterised by spiny projections termed acanthopodia (Page, 1967b). The ultrastructure has been described in a transmission electron microscopy study by Bowers & Korn (1968). They are typically uninucleate with a prominent nucleolus. They contain digestive vacuoles and a large water expulsion vacuole, which empties with a periodicity of 1-2 min. Lipid droplets and glycogen reserves are present in the cytoplasm. They also possess all the other typical organelles of a eukaryotic cell.

Trophozoites of *Acanthamoeba* are typically 25-40 μm in length, although there is much variation in length and shape due to locomotion (Page, 1967b). Movement is associated with the formation of pseudopods. At the front, which is usually broader than the rear, there is a wide hyaline zone termed the lobopodium. The amoeba progresses by gradual extension of the lobopodium forward over the surface. There are no brusque, eruptive movements, although one region may push ahead of the rest. A change in direction occurs when one side of the leading edge becomes dominant. Preston & King (1984) used reflexion interference microscopy to study the cell-substratum interactions involved in traction. They demonstrated the importance of a variable portion of the ventral surface of the amoeba, just dorsal to the lobopodium, which forms an 'associated contact' with the substratum from which thin extensions called filopodia are extended. Acanthopodia, which form cyclically on the lateral and dorsal surfaces, are also assumed to play a role in locomotion. The capability of trophozoites for movement, both on a planar surface and within a three-dimensional matrix, is obviously of great significance to their ability to invade the cornea.

Another important attribute of *Acanthamoeba* is that it can form cysts in adverse circumstances. The cyst stage is incredibly durable; it is able to withstand desiccation, temperature extremes and chemical insult, and may still be viable after 24 years (Mazur *et al.*, 1995). This has obvious implications for prevention of infection by contact lens sterilisation, and also for eradication of infection, both with regard to the efforts of the immune system and those of the clinician.

The cyst form of *Acanthamoeba* is a differentiating feature of the genus (Page 1967b). It is slightly smaller than the trophozoite (15-30 μm) and oval or triangulate in form. It is a double walled structure. The cellular contents are enveloped in a thin endocyst that is separated by a space from a fibrous, rugged external exocyst. The endocyst is polyhedral or stellate and adherent to the exocyst at certain points, termed ostioles, where the two layers form a plug or operculum. It is through these that the trophozoite emerges when the environment is again favourable. The sequential changes of the surface morphology which accompany encystation have been illustrated in a scanning electron microscopy (SEM) study (Pasternak *et al.*, 1970) The process of encystation has been arbitrarily divided into 3 stages, namely pre-encystation, cyst initiation and cyst wall formation. The ultrastucture of the cyst stage has been studied by transmission electron microscopy (Bowers & Korn, 1969) and the observed changes related to the process of encystation. Further understanding of the process has come from investigation of the molecular biology of the cell cycle and encystment (Byers *et al.*, 1991). Of particular interest is the discovery that diamidines are involved in induction of encystment. This has implications for use of dibromopropamidine (Brolene) in the treatment of this condition (Kinnear *et al.*, 1996; Kinnear & Hay, 1996).

Multiplication of *Acanthamoeba* occurs only in the trophozoite form. Asexual reproduction occurs by binary fission (Page, 1967b). During mitosis the nuclear membrane and nucleolus disappear (i.e. metamitosis). The molecular aspects of the cell cycle have been studied by Byers *et al.* (1991). There is no

convincing evidence for sexual reproduction as genetic recombination has not been demonstrated. Almost all knowledge concerning the *Acanthamoeba* genome has been derived from studies on the Neff strain of *Acanthamoeba castellanii* (see review by Byers *et al.*, 1990). Evidence suggesting that *Acanthamoeba* are polyploid is accumulating but is not yet conclusive. Nucleotide sequences for 5S and 5.8S rRNA have been determined directly from the RNA. Restriction fragment length profile studies of mitochondrial DNA reveal relatively high levels of overall sequence diversity. Such studies yield important information concerning phylogeny and taxonomy. They are also crucial to the design of molecular diagnostic probes.

The biology of *Vahlkampfia* and *Hartmannella* has not been investigated in similar depth although basic taxonomy studies have been performed (Page, 1967a & 1985). As with *Acanthamoeba*, in both cases the life cycle consists of only cyst and trophozoite forms. Members of the closely related *Hartmannella* genus are also characterised by a metamitotic pattern of cell division. They are monopodial, except temporarily, and move in a steady flowing fashion. Unlike *Acanthamoeba*, however, *Hartmannella* rarely form pseudopods and are elongated in locomotion. Also cysts of *Hartmannella*, and indeed of *Vahlkampfia*, do not form ostioles. In contrast, members of the *Vahlkampfia* genus are characterised by a promitotic pattern of nuclear division. They too are monopodial in locomotion, except temporarily, but move in a marked eruptive fashion. The above features are also demonstrated by members of the related genus *Naegleria*, which are distinguished by the presence of a flagellate stage in the life cycle.

It should be re-emphasised that *Acanthamoeba*, *Vahlkampfia* and *Hartmannella* are free-living amoebae. Their main source of nutrition is environmental bacteria, fungi, yeasts, algae and other small protozoa. They play an important role in nitrogen mineralisation (Weekers & van der Drift, 1993). Invasion of human tissue is purely opportunistic.

1.1.4 *Acanthamoeba* keratitis

The details below refer to *Acanthamoeba* keratitis. It is uncertain whether the clinical condition associated with *Vahlkampfia* and/or *Hartmannella* differs from *Acanthamoeba* keratitis, either in presentation or prognosis. Only as numbers of cases associated with the other two genera accumulate will comparisons be possible.

Pain is a particularly prominent symptom, often seemingly out of all proportion to the clinical signs and sometimes even requiring opioid analgesia (Kirkness *et al.*, 1994). *Acanthamoeba* keratitis should always at least be considered in the differential diagnosis of a contact lens wearer with a red eye, particularly if pain is a prominent feature. Elevated corneal lines (Florakis *et al.*, 1988) or a ring infiltrate (Theodore *et al.*, 1985) are also claimed to suggest the diagnosis, while radial keratoneuritis is considered virtually pathognomonic (Moore *et al.*, 1986).

The clinical presentation of *Acanthamoeba* keratitis is, however, remarkable for its varied manifestations. Several excellent review articles (Auran *et al.*, 1987; Bacon *et al.*, 1993a & 1993b; Kirkness *et al.*, 1994; Illingworth *et al.*, 1998) detail the complete spectrum of symptoms and signs so far recorded. The condition is most often confused with *Herpes simplex* keratitis (Johns *et al.*, 1987). Misdiagnosis is particularly likely early in the course of infection when the symptoms and signs are non-specific. This is compounded by the fact that *Acanthamoeba* keratitis often partially responds, at least temporarily, to a range of therapies. Concurrent infection also occurs (Bacon *et al.*, 1993b), potentially producing further diagnostic delay. Historically, therefore, *Acanthamoeba* keratitis has often been recognised late in the disease process.

For the first decade after emergence of *Acanthamoeba* keratitis as a clinical problem, treatment was typically unsatisfactory (Auran *et al.*, 1987). A wide

variety of topical agents were tried, often in combination, but penetrating keratoplasty was generally necessary. Even this may not be curative (Luxenberg, 1990), presumably due to retention of the organism in the corneal margins, and blindness or enucleation was a frequent outcome.

One of the first cases to be treated successfully medically (Wright *et al.*, 1985) utilised topical propamidine isethionate 0.1%. This was administered in combination with dibromopropamidine ointment 0.15% and followed by treatment with neomycin because toxicity occurred. Failure to eradicate the infection still commonly occurred, however, and more efficacious therapies were sought (Ficker, 1988).

Osato *et al.* (1991) recognised that the major barrier to therapeutic success was lack of amoebicidal effect once the organism has adopted the relatively protected cyst stage. Based on *in vitro* sensitivity studies demonstrating cysticidal activity, Larkin *et al.* (1992) reported a favourable outcome with the cationic disinfectant polyhexamethylene biguanide. Subsequent clinical experience with this compound in combination with propamidine has suggested that, if used early, then the prognosis is good and surgery is unlikely to be required (Bacon *et al.*, 1993a; Illingworth *et al.*, 1995; Linquist *et al.*, 1998). Alternatively, the related compound chlorhexidine, which also demonstrates cysticidal activity (Hay *et al.*, 1994), has produced favourable clinical outcomes when used in combination with propamidine (Seal *et al.*, 1996). Cysticidal activity may, however, be difficult to achieve in the deeper stromal layers using topical preparations. Even with relatively efficacious therapies, therefore, early diagnosis is of great relevance to the clinical outcome (Bacon *et al.*, 1993a; Illingworth *et al.*, 1995; Lindquist, 1998).

The use of confocal microscopy to demonstrate the organism *in situ* in the cornea may be of benefit in rapid clinical diagnosis (Chew *et al.*, 1992 & Winchester *et al.*, 1995). Indeed, Mathers and colleagues (1996) report the use

of this technique for routine screening of all patients presenting with keratitis. In their study the diagnosis of *Acanthamoeba* keratitis was based on the presence of ovoid refractive bodies in the cornea and subsequent cytological identification of amoebae in epithelial smears. The authors recorded an increased incidence of *Acanthamoeba* keratitis, over half of which occurred in non-contact lens wearers. They therefore suggested that *Acanthamoeba* keratitis is' underdiagnosed. This would imply that many cases are misdiagnosed or overlooked due to self-limiting infection or resolution on non-specific therapy (Illingworth *et al.*, 1995). It is my contention that this postulated self-limitation of some cases may be related to confinement of *Acanthamoeba* to the epithelium. Indeed, early in infection epithelial debridement alone may be curative (Brooks *et al.*, 1994). This would at least partially explain the apparent importance of early diagnosis and rapid initiation of therapy. It also has implications for diagnosis based on epithelial scrapes alone (Section 1.2.3) and for the pathogenesis of the condition (Section 1.3.5).

The management of *Acanthamoeba* keratitis is further complicated by the fact that it classically follows a relapsing and remitting course. Moreover, *Vahlkampfia* and *Hartmannella* keratitis may theoretically be associated with a suboptimal response to classical anti-*Acanthamoeba* therapy (Aitken *et al.*, 1996). In order to improve medical management of amoebal keratitis, suitable *in vitro* models are required for testing of potential chemotherapeutic agents. Apart from simple assessment of their ability to kill cysts and trophozoites, it would be desirable to test candidate pharmacological compounds with respect to toxicity for corneal cells and their efficacy in eradicating infection in corneal tissue.

1.2 Laboratory Diagnosis

1.2.1 Introduction

In general, laboratory diagnosis of any infectious disease is based, directly or indirectly, on demonstration of the presence of an infectious agent capable of

producing the observed pathology. This is followed by identification of genus, species and/or strain of organism involved as appropriate, thus facilitating selection of appropriate treatment and providing information concerning likely prognosis.

To demonstrate the presence of amoebae, a sample of corneal tissue is obtained either by debriding the epithelium (Section 1.2.3) or by a punch biopsy technique (Kirkness *et al.*, 1994). The latter may be required in a late-presenting case if the initial epithelial scrape is negative, as the infection is likely to have advanced to stromal invasion. The specimen is removed from the presumed site of advancing infection (i.e. at the edge of the ulcer) as the area of greatest inflammation may, in fact, be sterile. Difficulties encountered in identifying the presence of amoebae are discussed below (Section 1.2.3). Also the occurrence of mixed infections (Beattie *et al.*, 1990, Aitken *et al.*, 1996) may lead to confusion in laboratory diagnosis.

Due to the ubiquitous nature of amoebae, the potential for specimen contamination must always be considered. For an unequivocal laboratory diagnosis two separate positive results are preferable. Specimens are therefore sent both for culture (Section 1.2.2) and for pathological examination using a range of techniques (Section 1.2.3). It is desirable that a member of the laboratory staff is present at the time of sampling to ensure optimal practice. All equipment used, including glass slides, must be clean and indeed sterile if intended for culture.

As described above (Section 1.1.2) speciation of amoebae is confused and currently under review. In the majority of ocular microbiology units, therefore, amoebae derived from clinical specimens are not routinely speciated. It is, however, desirable to distinguish at least the genus of amoeba as this may influence selection of appropriate antiprotozoal therapy: *in vitro* drug sensitivity studies suggest that *Vahlkampfia* and *Hartmannella* are more

sensitive to amphotericin B than to chlorhexidine (Aitken *et al.*, 1996). Identification of the genus may also yield clinically relevant information concerning differences in the presentation, progression and prognosis of amoebal keratitis due to different genera of amoebae. Differentiation of the 3 genera of amoebae may, however, be difficult in practice (Section 1.2.3). The potential of molecular methods for differential diagnosis is also discussed (Section 1.2.4).

1.2.2 Microbiology

Agar culture is the mainstay for laboratory detection of amoebae. Non-nutrient agar is seeded with heat-killed bacteria (both Appendix A). The specimen is inoculated on to a delineated area and the surface moistened with amoebal saline (Appendix A). Plates are incubated in air at 25 ^{0}C and 32 ^{0}C as different species thrive at different temperatures (Kelly & Mitra, 1993). The plate, which need not be opened, should be examined on a daily basis using an inverted microscope. Confident identification requires experience; it may be helpful to incubate an uninoculated control plate. Snail-like trails on the surface of the agar indicate migration of amoebae. Agar crystals or bubbles in the agar must not be misinterpreted as cysts or trophozoites respectively. Careful observation will demonstrate typical morphology with a double cell wall and motility with the presence of a contractile vacuole (periodicity - 2 min) for cysts and trophozoites respectively. The clinical sample may also be inoculated into a suspension of heat-killed bacteria in a small tissue culture flask. Furthermore, if specialised culture techniques are not available on site, specimens may be inoculated into a transport medium such as amoebal saline and sent by mail; concentration of the sample should be performed by filtering onto a membrane (Gradus *et al.*, 1989) or by centrifugation (personal experience). This should not adversely affect the ability to isolate the organism as trophozoites will simply encyst under adverse conditions and cysts will not be readily harmed.

Isolation on culture is the gold standard but identification of the genus of amoebae involved generally requires an experienced protozoologist. Ocular

pathological specimens are also particularly small and the organisms concerned may be difficult to isolate (Auran *et al.*, 1987). Warhurst & Mann (1988) suggest that incubation should be continued for 5-6 days only. Many of the patients under investigation, however, have been pretreated; it is perhaps for this reason that experience in the Tennent Institute suggests that prolonged incubations (eg 2-4 weeks) may be necessary. Such a delay in diagnosis is obviously undesirable. Isolation on culture does, however, allow assessment of sensitivities to potential antimicrobial drugs although, in the case of amoebal keratitis, this is not routinely performed.

1.2.3 Histology and Immunodiagnosis

Unlike culture, histology permits the demonstration of cysts and trophozoites *in situ* in the tissue, minimising the risk of false positive results due to contamination. The contact lens may also be examined by light microscopy (Johns *et al.*, 1989; Johns *et al.*, 1991) or by scanning electron microscopy (Kirkness *et al.*, 1994). Similarly fluid from the contact lens case may be cultured or examined by phase contrast microscopy (Bottone *et al.*, 1992b). The presence of amoebae does not, however, necessarily imply that they are the causative agent of disease. This was highlighted recently by Newman *et al.* (1997) who reported a case of keratitis where, although *Acanthamoeba* was isolated from an epithelial scrape, it was considered likely that the organism was a transient, rather than responsible for the observed ulcerative process. Furthermore, as discussed in Section 1.1.4, mild self-limiting infection may be associated with confinement of *Acanthamoeba* to the epithelium. The significance of the presence of an amoeba in an epithelial scrape only is thus open to question in my opinion.

In spite of these provisos, direct wet mount examination of epithelial scrapings has proved useful for diagnosis of *Acanthamoeba* keratitis (Singh & Sachdeva, 1994), especially using phase-contrast microscopy (Fig 1.4). As for culture, double-walled cysts or motile trophozoites with contractile vacuoles may be observed, features that facilitate differentiation from other cells present. It is

our laboratory policy to regard such a positive result as provisional until confirmed by an alternative method. This method, and also light microscopy of the contact lens and contact lens case fluid, do, however, have the advantage of speed thus facilitating rapid commencement of appropriate therapy with the attendant benefits regarding prognosis (see Section 1.1.4).

Specimens may also be processed for histology by light or transmission electron microscopy. Haematoxylin and eosin (H&E - Theodore *et al.*, 1985), Giemsa (Moore *et al.*, 1985; Theodore *et al.*, 1985; Wilhelmus *et al.*, 1986; Auran JD *et al.*, 1987; Ficker, 1988; Kirkness *et al.*, 1994), periodic acid schiff (PAS - Theodore *et al.*, 1985; Ficker, 1988), Grocott-Gomori methenamine silver (Theodore *et al.*, 1985; Ficker, 1988; Kirkness *et al.*, 1994), acridine orange (Kirkness *et al.*, 1994), Wright stains (Kirkness *et al.*, 1994) Trichrome (Theodore *et* al, 1985), Gram (Theodore *et al.*, 1985; Wilhelmus *et al.*, 1986; Ficker, 1988; Kirkness *et al.*, 1994), Hemacolour (Theodore *et al.*, 1985), lactophenol cotton blue (Thomas *et al.*, 1990), modified Papanicolaou stain (Karayianis, 1988), calcofluor white (Wilhelmus *et al.*, 1986; Marines *et al.*, 1987; Silvany *et al.*, 1987) and fluorescein-conjugated lectins (Robin *et* al, 1988; Robin *et al.*, 1989) have all been suggested to be of some limited benefit in the diagnosis of *Acanthamoeba* keratitis. H&E, Giemsa and PAS are commonly used for this purpose in routine pathology laboratories. Their efficacy for diagnosis of *Acanthamoeba* keratitis had not been compared with the other methods mentioned.

Calcofluor white in particular is a rapid and simple technique that also has potential for visualisation of fungal elements (Sutphin *et al.*, 1986). As such it is worth considering as a first line approach to epithelial smear preparations in which both fungal and amoebal infection are under consideration. Quenching of fluorescence occurs with time although stored slides can be rapidly restained (Wilhelmus *et al.*, 1986). Furthermore, following destaining, the technique can also be applied to archival slides if no unstained sections are available (Silvany *et al.*, 1987).

In spite of the characteristic prominent nucleolus of amoebae (John, 1993), with such non-specific staining methods, difficulties may be encountered in distinguishing trophozoites from the associated non-specific inflammatory cell infiltrate (Mathers *et al.*, 1987; Garner, 1993), and from keratocytes and epithelial cells (Fig 1.5). Although cysts are more readily identified, preservation of morphology is often poor in clinical specimens: small, delicate tissue samples (1-2 mm) are easily traumatised post-biopsy while inflammation and treatment *in situ* also contribute. Transmission electron microscopy yields greater morphological detail but again interpretation may be difficult, particularly if the plane of section does not include the nucleus.

Even if amoebae are identified, differentiation of the 3 genera on the basis of morphology is virtually impossible in view of the above factors. Also fixed specimens do not permit observation of characteristic locomotor patterns or nuclear division. More specific labelling methods are therefore desirable to identify the presence of amoebae in such specimens and also to distinguish the genus involved.

Unlike conventional staining methods, immunocytochemistry techniques rely on recognition of a target antigen by the complementary antibody to produce specific labelling, tagged by a microscopically visible label. Polyclonal antisera raised against *Acanthamoeba* have therefore been used to attempt to overcome problems in identifying the presence of amoebae in pathological specimens (Blackman *et al.*, 1984; Epstein *et al.*, 1986). It has been suggested, however, that cross-reactivity occurs, at least with the antiserum in routine use at the Tennent Institute (Dr D.V. Seal - personal communication) producing labelling of corneal tissue, particularly epithelial cells and of immune cells, especially macrophages. These are claimed to be pivotal to the host defence (van Klink *et al.*, 1996) and presumably may contain intra-cellular amoebal debris. Such postulated cross-reactivity requires investigation.

Raised against *Acanthamoeba* and putatively specific for *Acanthamoeba*, the cross-reactivity of these antisera has also not been tested against the other genera of free-living amoebae presently under study as they had not previously been implicated in disease. Possession of common epitopes with cross-reactivity of antisera has, however, been shown in other studies of closely related amoebae (Stevens *et al.*, 1977). Such cross-reactivity would lead to erroneous identification and may have contributed to previous failure to appreciate the involvement of genera other than *Acanthamoeba*. Alternatively, if the antisera are indeed specific for *Acanthamoeba*, cases of amoebal keratitis associated with other genera of amoebae would produce false negative results. This potential cross-reactivity requires investigation.

1.2.4 Molecular Biology Techniques

Specific recognition of target sequences is also the basis of molecular biology diagnostic techniques. A number of features make these methodologies particularly attractive for diagnosis of ocular infections (Kinnear & Kirkness, 1995). Unlike antibody production, whether for PCR or ISH, a probe complementary to the desired target sequence is relatively easily manufactured. Furthermore, selection of a probe sequence and assessment of its potential specificity is facilitated by genebank data and programs that allow comparison with all other known deoxyribonucleic acid/ribonucleic acid (DNA/RNA) sequences.

The PCR procedure, which involves exponential multiplication of a target sequence, requires only a small sample, with no need for elaborate arrangements for transportation or preparation. It is also a rapid procedure which may be completed in less than 7 hr; being essentially mechanised it is non-labour intensive and can be performed overnight. These factors suggest that PCR might be usefully exploited for diagnosis, particularly where i) a rapid, sensitive test is required, ii) the sample size is particularly small, or iii)

transportation or preparation requirements for other procedures are difficult to implement in practice (eg in developing countries). Amoebal keratitis is thus a condition for which this technology may be appropriate. Several different primers have already been described in the literature (Vodkin *et al.*, 1992; Lai *et al.*, 1994; Gast *et al.*, 1995), although, at the time of writing, none is in routine diagnostic use.

For the present study PCR Primers P1 and P2, putatively specific for the genus *Acanthamoeba,* were selected from the literature (Lai *et al.*, 1994). The primers chosen had been designed to recognise part of the 925 base pair (bp) BamHI-SstI fragment of *Acanthamoeba castellannii* 26S ribosomal deoxyribonucleic acid (rDNA - EMBL accession number X73881). This 126-bp BamHI-SstI restriction fragment (nucleotides 1-126) of the 925-bp 26S rDNA locus is termed ArDNA (126-bp) and is part of a variable region of the rDNA (Lai *et al.*, 1993).

From the point of view of differential diagnosis of *Vahlkampfia* and *Hartmannella* keratitis, primers for the specific detection of *Hartmannella* and *Vahlkampfia* are not currently available in the literature. Furthermore primers P1 and P2 have not been tested against these particular genera. It is therefore important to assess whether these primers are indeed specific for *Acanthamoeba*, or whether they are also capable of detecting members of the *Hartmannella* and *Vahlkampfia* genera. Following optimisation of the protocol the sensitivity of these primers requires to be assessed, as does their potential for diagnostic purposes in the presence of corneal tissue.

Although ISH had not previously been described for the demonstration of amoebae, this technique may, in future, be a more appropriate technique for the diagnosis of amoebal keratitis. Considering the ubiquitous presence of *Acanthamoeba* and related free-living amoebae in the environment, and the fact that the end result is the presence or absence of a band on a gel, the

potential for false positive results with PCR is obvious. In contrast, the ISH method, which involves recognition of a target sequence with complementary labelled DNA, is a less sensitive method but has the advantage of demonstrating the organism *in situ* in the biopsy specimen.

The ability of the labelled 126-bp probe complementary to the PCR product to demonstrate *Acanthamoeba castellanii* in corneal tissue therefore requires investigation. Cytospin preparations and inoculated corneas may be used to represent epithelial scrape and corneal biopsy specimens respectively, with optimisation of a standard ISH protocol for these applications. The potential cross-reactivity with *Vahlkampfia* and *Hartmannella* also requires to be assessed.

1.3 Pathogenesis

1.3.1 Introduction

The potential to produce established infection depends on the balance between the ability of the organism concerned to invade and survive within host tissues, and the ability of the host to prevent this. Any resulting disease is a combination of both these variables, which renders investigation of pathogenesis complex. Models of a disease, both *in vitro* and in animals, are often utilised to assist understanding. They are, by definition, imperfect and findings must be interpreted cautiously, with due recognition of how the model differs from the condition it is representing.

Histology of corneal tissue not only facilitates diagnosis but also provides information concerning the associated pathology and hence the pathogenesis of *Acanthamoeba* keratitis. Epithelial scrapes, corneal biopsy specimens, keratoplasty discs and evisceration specimens have all been analysed. A summary of the results of a number of studies (Garner, 1993; Blackman *et al.*, 1984; Mathers *et al.*, 1987) is outlined below (Section 1.3.2). These are

compared to the findings in animal models, with consideration of their relevance to the understanding of the human condition (Section 1.3.3).

It has been demonstrated that a proportion of healthy individuals possess IgG antisera against *Acanthamoeba*, suggesting that subclinical infection has occurred (Cursons *et al.*, 1980). *Acanthamoeba* has also been isolated from the normal commensal flora of the respiratory tract (Wang & Feldman, 1967). It has also been shown to be present in the contact lens case (Larkin *et al.*, 1990 - 7%; Devonshire *et al.*, 1993 and Clark *et al.*, 1994 - 4%) and even the tear film (Dr John Hay - personal communication) without evidence of keratitis. Humans thus frequently come in contact with these ubiquitous organisms without developing overt infection.

Acanthamoeba is essentially an opportunistic pathogen that requires a range of favourable circumstances before contact with the organism results in disease (Martinez & Janitschke, 1985). With regard to both the host and the role of the contact lens, these facilitating factors are only partially understood. As already alluded to, it is unknown whether the ability to produce disease is restricted to a subset of *Acanthamoeba*, or whether all species are potentially pathogenic. Invasion of the organism requires the ability to adhere to and then penetrate the corneal epithelium. To produce ongoing infection it must also be able to survive within the stroma while evading the immune response. The facility to do this is related to virulence factors that are also only partially understood.

The pathogenesis of *Acanthamoeba* keratitis is therefore discussed with respect to the relative contribution of facilitating host factors (Section 1.3.4) and amoebal virulence factors (Section 1.3.5). Whether the findings are relevant to *Vahlkampfia* and *Hartmannella* keratitis awaits recognition of more cases and elucidation of the biology of these little researched organisms.

1.3.2 Pathology: Human *Acanthamoeba* Keratitis

In keratoplasty specimens of patients with *Acanthamoeba* keratitis the epithelium is often necrotic or absent, with ulceration of the underlying stroma (Garner, 1993). Re-epithelialisation of previously ulcerated tissue may also be observed. Bowman's layer may be present or absent. Neovascularisation does not generally occur. Cysts and/or trophozoites may be confined to the epithelium plus/minus the anterior stroma, or scattered throughout the stroma (Fig 1.2B & 1.3B). They are generally associated with an inflammatory infiltrate of neutrophil polymorphonuclear leucocytes and mononuclear cells, although in the deeper layers this may be less marked or absent (Fig 1.5). Lymphocytes and plasma cells are rare. These findings have been confirmed by immunocytochemical staining (Mathers *et al.*, 1987). There may be associated corneal thinning, again particularly in the anterior stroma; not associated perforation has also been described. These differing appearances are assumed to be related to progression of the condition (Garner, 1993).

The corneal epithelial cells and keratocytes may appear degenerate, as may the inflammatory cells and indeed, the amoebae themselves. The observed damage could theoretically be due to the effects of i) the amoebae, ii) the immune system iii) therapy or iv) any combination of the previous 3.

In some cases, although amoebae are scattered throughout the cornea, the immune cell infiltrate and necrosis of tissue is largely confined to the anterior stroma. Keratocyte depletion is also observed; this, however, may extend throughout the stroma even when the inflammatory infiltration is confined to the anterior layers. It has therefore been suggested that the tissue necrosis is largely associated with the inflammatory reaction while the amoebae themselves are responsible for loss of keratocytes (Garner, 1993). This author further suggests that the sequence of events may be as follows: i) Parasitic invasion of the anterior stroma occurs secondary to an epithelial defect. ii) Trophozoites spread throughout the cornea, depleting it of keratocytes. iii) In response an inflammatory reaction occurs, resulting in stromal necrosis

secondary to leucocyte and parasitic collagenolysis. It has, however, also been demonstrated that traumatic damage to corneal epitheium may itself induce keratocyte depletion (Campos *et al.*, 1994; Wilson *et al.*, 1996).

Another feature of the disease is that inflammation commonly worsens on commencement of therapy. It has been suggested that death of the organism may release antigens that were previously hidden. (Blackman *et al.*, 1984; Lindquist *et al.*, 1990). Alternatively increased inflammation may be related directly to spillage of intracellular contents; collagenase produced by a corneal isolate of *Acanthamoeba castellanii*, when injected into the stroma of Lewis rats' corneas, has been shown to produce a keratitis similar to that produced by the organisms themselves (He *et al.*, 1990).

With regard to ocular damage, the relative contributions of the amoebae, and the efforts of the immune system and the clinician to eradicate the organism, have important implications for management. This requires to be further assessed. In spite of their inherent deficiencies, the use of *in vitro* models is particularly desirable in this context as individual variables can be altered independently.

1.3.3 Pathology: Animal Models of *Acanthamoeba* Keratitis

Animal models demonstrating successful invasion of cornea by *Acanthamoeba castellanii* have been established in the pig (He *et al.*, 1992) and the Chinese hamster (van Klink *et al.*, 1993). These involved application of an amoeba-laden contact lens to an abraded corneal surface followed by tarsorrhaphy. The same procedure did not induce keratitis in rabbits (He *et al.*, 1992). The choice of host was based on earlier *in vitro* studies suggesting that only human, pig and Chinese hamster corneas were susceptible to invasion by *Acanthamoeba castellanii*, while the amoebae did not even bind to the epithelial surface of those of rabbit, rat and various other animals (Niederkorn *et al.*, 1992). An *in vivo* rabbit model of *Acanthamoeba* keratitis has been described but this involved direct inoculation of the *Acanthamoeba castellanii* into the corneal stroma (Font *et al.*, 1981 & 1982, Cote *et al.*, 1991). Rat models have also been

developed using inoculation of *Acanthamoeba polyphaga* alone (Larkin & Easty, 1990a & 1991) or by co-inoculation of various strains *Acanthamoeba* and *Corynebacterium* (Badenoch *et al.*, 1990).

Histological studies on the rat model involving inoculation of *Acanthamoeba polyphaga* only (Larkin & Easty, 1990a) have demonstrated liquefactive stromal necrosis with *Acanthamoeba* within the stroma persisting at 70 days. A brisk inflammatory response was observed in the early days of infection but declined thereafter. The severity of the inflammatory reaction correlated with the severity of the keratitis, which was also more marked initially. Clinically this consisted of a granular opacity, with corneal thinning superficial to infiltrates as the infection progressed. Vascularisation was minimal and perforation was not observed. There was also no evidence of anterior uveitis.

Immunohistochemical studies (Larkin & Easty, 1991) demonstrated that the cellular response consisted solely of neutrophils on the first day but macrophages appeared on the following days, becoming the predominant cell type by day 7. Some T, but no B, lymphocytes were observed; these first appeared on day 7. These findings largely agree with the results of pathological studies of human *Acanthamoeba* keratitis. No epithelial disease was evident, presumably because the amoebae had been inoculated intrastromally. In the other rat model, involving co-inoculation of *Corynebacteria*, the time course and histological findings were similar, but peripheral neovascularisation and microabscess and hypopyon formation were also observed in some cases (Badenoch *et al.*, 1990). Histopathological examination was not described in any detail for the rabbit model but hypopyon formation has been reported (Cote *et al.*, 1991) as has ulcerative keratitis (Font *et al.*, 1981).

The pig (He *et al.*, 1992) and Chinese hamster (van Klink *et al.*, 1993) models of keratitis are perhaps more relevant to the human disease in that the amoebae gain access to the stroma by epithelial invasion. Prior epithelial abrasion was a

prerequisite for establishing infection. Epithelial ulceration and corneal opacity secondary to oedema and inflammatory cell infiltrate consisting predominantly of neutrophils are described for both models. More advanced infection in the pig model was associated with characteristic ring-like infiltrates, epithelial microcysts and stromal thinning with aqueous flare and keratitic precipitates. In contrast to human infection, however, neovascularisation occurred in both models. The condition was also self-limiting: resolution with residual scarring occurred within 8 weeks in the pig and only 2 weeks in the Chinese hamster.

Hence animal models of *Acanthamoeba* keratitis have yielded useful information. It has not, however, been possible to develop an animal model promoting the development of keratitis simply by placing a parasite-laden contact lens onto an intact corneal surface. This is commonly interpreted as inferring that an epithelial defect is required to allow the amoebae to gain access. It should also be noted that the human condition may involve a prolonged phase when infection is limited to the epithelium involvement only, while in animal models the condition is predominantly stromal. Development of an *in vitro* model to investigate the process of amoebal invasion is therefore desirable.

1.3.4 Facilitating Factors

Pathogenicity may be determined as much by ability to adapt to the environment and evade the host defence mechanisms as by specific virulence factors. For example, Griffin (1972) investigated the role of temperature tolerance for pathogenic and non-pathogenic free-living amoebae (*Naegleria, Acanthamoeba, Hartmannella* and *Tetramit us*). Although growth at high temperatures (40 ^{0}C) indicated a potential for neural pathogenicity, each strain had to be tested on mice, suggesting additional barriers to pathogenicity other than temperature sensitivity. A study of 19 species of *Acanthamoeba* (De Jonckeere, 1980) also suggested that, although growth at high temperatures and readiness to grow axenically indicated a potential for pathogenicity, each strain had to be tested in cell cultures or mice to determine whether or not it was

virulent (i.e. capable of producing fatal neural infection). Thus a variety of factors related to the host environment determine whether a particular isolate is able to invade and produce disease.

In contrast to the above results, a further study involving ocular isolates of *Acanthamoeba* suggested that a positive correlation with growth at high temperature does not exist for ocular isolates (De Jonckheere, 1991). In this regard it should be noted that potential pathogenicity for neural and ocular tissues may differ. For example growth in corneal tissues would require relatively little temperature tolerance (Efron *et al.*, 1989). Indeed, Visvesvara *et al.* (1975) have demonstrated that an ocular isolate of *Acanthamoeba polyphaga* produced a cytopathic effect on cultured cells but did not produce meningoencephalitis in mice. Thus, an organism may be pathogenic with respect to one particular environment within a host and not another. It does not, therefore, seem appropriate to use the ability to produce meningoencephalitis in mice as an indicator of ocular pathogenicity. The following discussion is directed towards factors that facilitate amoebal keratitis.

In vivo the intact corneal epithelium is generally assumed to represent an effective barrier against amoebal invasion. Indeed, as discussed in Section 1.1.4, mild, self-limiting infection may be associated with early *Acanthamoeba* keratitis when the organism is confined to the epithelium. Evidence from animal models (Section 1.3.3) suggest that an epithelial defect is a prerequisite for invasion. The association with contact lens wear is generally postulated to be due to epithelial compromise and microtrauma (Moore *et al.*, 1987). Theoretically, protection of invading amoebae from the wiper action of the eyelids by the contact lens may also play a role.

Furthermore, fungal, and more particularly bacterial, co-contaminants within the contact lens care system (Fig 1.6) are suggested to support growth of

Larkin et al., 1990)
Acanthamoeba (Donzis *et al.*, 1989 This increases the available inoculating dose and thus may be an important prelude to infection (Bottone *et al.*, 1992a). Similarly, *Corynebacteria* were successfully utilised as an adjunct in the establishment of a rat model of *Acanthamoeba* keratitis, when inoculation of amoebae alone failed to produce keratitis (Badenoch *et al.*, 1990). In this scenario the inoculating dose is constant, but the bacteria may support trophozoite growth in the initial stages of infection. They may also facilitate penetration by contributing to tissue damage. It has also been suggested that the external eye flora, which are known to show significant quantitative changes in the commensal bacteria in asymptomatic contact lens wearers, may act as a nutrient source and hence play a role in the pathogenesis of *Acanthamoeba* keratitis (Larkin & Easty, 1990; Larkin & Leeming, 1991).

Culture for bacteria and fungi is often performed in addition to amoebal culture in cases of suspected *Acanthamoeba* keratitis. In one large series, bacteria were isolated in a total of 20 of 72 cases of *Acanthamoeba* keratitis, 14 on initial corneal scraping and in 11 cases on subsequent rescraping (Bacon *et al.*, 1993b). Co-infection is therefore by no means a prerequisite for *Acanthamoeba* keratitis The exact significance of co-isolates is also often uncertain. In some cases an initial co-infection may have occurred, presumably facilitating amoebal pathogenicity, but in other cases bacterial co-infection may have been secondary, particularly where there was a chronic epithelial defect (Bacon *et al.*, 1993b).

In contrast to chronic granulomatous encephalitis, corneal infection with these opportunistic pathogens occurs in individuals who have a normal immune system. *Acanthamoeba* keratitis, however, is associated, not with haematogenous spread, but with direct invasion of the cornea. It has therefore been suggested that the relatively poor immunoprotection of the cornea may predispose to infection at this site. The eye, like the brain, is an immunopriviledged site. Although infiltration of inflammatory cells does occur, as described above for both the human condition and animal models,

this predominantly consists of neutrophils with some macrophages and few lymphocytes. The relative lack of an effective second-line, parasite-specific response presumably facilitates both chronicity and recrudescence of *Acanthamoeba* keratitis.

With regard to involvement of non-corneal tissues, moderate to severe chronic uveitis, most evident anteriorly, has been described in eyes enucleated due to *Acanthamoeba* keratitis. This was composed of diffuse lymphocyte and plasma cell infiltrates and associated with vascular congestion and chronic inflammation of the perilimbal conjunctiva (Garner, 1993). Panophthalmitis with retinal detachment has also been described secondary to *Acanthamoeba* keratitis (Key *et al.*, 1980; Burke *et al.*, 1992). The former case was associated with optic nerve oedema, while the latter demonstrated optic nerve atrophy. Disc swelling has also been demonstrated by computed tomography scan in a patient with this condition (Mannis *et al.*, 1986). In none of these cases, however, were amoebae demonstrated in tissues other than the cornea, suggesting that the inflammation was a sterile secondary response to anterior inflammation. This is well recognised as a general phenomenon. It has been documented in *Acanthamoeba* keratitis (Mannis *et al.*, 1986) and has even been suggested to result in sceral ectasia, presumably secondary to collagenolysis, if sufficiently prolonged (Lindquist *et al.*, 1990).

Four cases of proven *Acanthamoeba* infection of non-corneal ocular infection have, however, been described (Jones *et al.*, 1975; McClellan & Coster, 1987; Dougherty *et al.*, 1994; Heffler *et al.*, 1996). Two of these are associated with *Acanthamoeba* infection at other sites with ocular involvement presumed to be secondary to haematogenous spread (Jones *et al.*, 1975; Heffler *et al.*, 1996). The most recent case (Heffler *et al.*, 1996) involved an immunocompromised patient with disseminated *Acanthamoeba* infection of the skin and lungs. Endophthalmitis with iris granulomas and choroidal infiltrates was documented clinically and the organism was isolated from the aqueous humor and the vitreous; the patient subsequently died but an autopsy was not performed.

Similarly, in the earliest case (Johns *et al.*, 1975) choroiditis was associated with *Acanthamoeba* meningoencephalitis secondary to pharyngitis. Blood borne infection was also postulated in another case of chorioretinitis, this time in the contralateral eye of a patient with *Acanthamoeba* keratitis (Johns *et al.*, 1988). This assertion, however, is based purely on the clinical observation of yellow-white deposits in the retina associated with a subretinal creamy-yellow cystic lesion. Similar lesions have been described in patients with *Enolimax nana, Entamoeba coli* and *Dientamoeba fragilis* (Knox & Bayless, 1957). In none of these cases were amoebae demonstrated *in situ*, and thus a secondary sterile immune reaction is also a possibility (see Kinnear *et* al, 1995 for discussion of possible mechanisms).

Thus, only two of these cases of proven non-corneal ocular infection concerned direct invasion from a primary corneal infection (McClellan & Coster, 1987; Dougherty *et al.*, 1994). In the former case *Acanthamoeba* was demonstrated in an aqueous tap, but no inflammation of the posterior segment is documented. In the latter case scleral nodules that were positive for *Acanthamoeba* on culture are described; enucleation was subsequently performed and histopathological examination revealed an *Acanthamoeba* cyst associated with a granulomatous inflammatory response deep in the sclera. Although both these cases involved contact lens wearers who were otherwise healthy, they were associated with advanced keratitis that had been treated with steroids. Steroids have a well-recognised immunosuppressive effect that would presumably enhance amoebal pathogenicity. Indeed, work in a rabbit model of keratitis by John *et al.* (1991) suggests that *Acanthamoeba* are more pathogenic when inoculated in the presence of corticosteroids.

This paucity of proven cases of non-corneal infection with *Acanthamoeba* implies that the organism is usually effectively confined to the cornea. This, combined with the fact that the few cases that are documented are associated with haematogenous spread and/or immunosuppression, suggests that penetration of the organism past the limbus is generally prevented, in spite of

the relatively poor ocular immune system. This suggests that factors specific to the cornea are responsible for both facilitating and limiting amoebal pathogenicity.

It is of note that the cornea is (normally) avascular. Interestingly, even chronic *Acanthamoeba* keratitis rarely produces vascularisation as would be expected following prolonged inflammation. Furthermore, in the Chinese hamster and pig models, neovascularisation does occur and is associated with self-limiting infection (Section 1.3.3). Vascularisation, while detrimental to vision, facilitates more effective immunoprotection. There is also a relative lack of antigen-presenting cells in the cornea. This may be at least partially responsible for the observed failure of effective recruitment of IgG or delayed type hypersensitivity responses. In the Chinese hamster model it has been shown that the presence of Langerhans cells in the central cornea (induced to migrate by latex beads or IL-1 pretreatment) produced significant protection against Acanthamoeba infection (van Klink *et al.*, 1993). Furthermore, in the one case of documented scleral infection, histopathology demonstrated an intense inflammatory reaction with lymphocytes, plasma cells, histiocytes and multinucleate giant cells.

Presumably on the basis of their ability to produce encephalitis, it has been suggested that free-living amoebae demonstrate a predilection for neural tissue. Indeed, the olfactory nasal epithelium is the presumptive portal of entry (Martinez, 1983; Ma *et al.*, 1990; John, 1993) primary amoebic meningoencephalitis due to *Naegleria*, as has been demonstrated experimentally in mice (Martinez *et al.*, 1973). Furthermore, Marciano-Cabral *et al.* (1982) have demonstrated that *Naegleria fowleri* is more cytopathic for cultured neuroblastoma cells than for other tested cell lines. Neural infection is also described secondary to intranasal inoculation of *Acanthamoeba* in mice (Culbertson *et al.*, 1958). The association with the neural environment may, however, be due as much to the relative immunoprotection as to any advantage of neural cells as a food source.

With respect to non-corneal ocular tissue, experimental optic neuritis and uveitis have been described in a rabbit model (Schaegel & Culbertson, 1972). Following intra-vitreal injection, although the anterior chamber was affected, the inflammation was most severe in the choroid and retina with optic nerve involvement; *Acanthamoebae* were demonstrated within the retina and the optic nerve. An amoeba was also demonstrated in the optic nerve of one animal that had received drops of amoebae suspension onto the cornea only.

With respect to the cornea, radial keratoneuritis, an unusual clinical sign associated with *Acanthamoeba* keratitis, has been suggested to be due to amoebal infiltration of the corneal nerves (Moore *et al.*, 1986). Corneal nerve involvement is generally assumed to be the underlying cause of the severe pain commonly associated with the condition. It is, however, unknown whether the infiltrates consist of inflammatory cells and/or the amoebae themselves. Insler *et al.* (1988) were unable to demonstrate the presence of inflammatory cells or neural cells on histology and therefore suggest that the lines may simply represent the path of migrating amoebae. Pfister *et al.* (1996), however, claim to have demonstrated the presence of a trophozoite adjacent to a swollen nerve fibre by confocal microscopy. Theoretically the corneal nerves may facilitate invasion.

Thus, various factors associated with contact lens wear are of great significance in determining the potential of free-living amoebae to produce opportunistic infection of the cornea. The ability of amoebae to produce keratitis is also potentiated by the general immunopriviledge associated with the eye in combination with features specific to the cornea. The facilitating factors relevant to pathogenesis are, however, still only imperfectly understood. While studies on animal models have the advantage of being performed *in vivo* in the presence of immune surveillance, they fail to mimic the human condition in important ways. Further investigations on *in vitro* models utilising human corneas are therefore required to supplement animal studies. It is also

appropriate to compare the relative pathogenicity of *Acanthamoeba, Vahlkampfia* and *Hartmannella* on human tissue models.

1.3.5 Amoebal Pathogenicity and Virulence

Adherence is a prerequisite for invasion. It has been shown that trophozoites of various species can bind to human corneal buttons *in vitro* (McLaughlin *et al.*, 1991). This is also the case for various animal corneal buttons (Niederkorn *et al.*, 1992); indeed the choice of the Chinese hamster and pig for animal models was based on such data. Scanning and transmission electron microscope studies of this process suggest that the attachment regions are characterised as plaque-like maculae of an incomplete desmosomal junction (Ubelaker *et al.*, 1991). *In vitro* experiments have shown that *Acanthamoeba* can bind to the extracellular matrix proteins collagen, laminin and fibronectin, and that the adherence process is inhibited by mannose (Gordon *et al.*, 1993). It is unclear whether isolates of proven virulence show enhanced binding to human cornea, as has been suggested (Morton *et al.*, 1991a). In another study by the same group, however, adhesion of amoebae to rabbit corneal epithelium did not consistently correlate with the reported pathogenicity of the strain of *Acanthamoeba* (Morton *et al.*, 1991b).

Post-binding, invasion is the next essential step in the establishment of infection. Incubation of corneal buttons with *Acanthamoeba castellanii* resulted in severe epithelial ulceration with penetration of trophozoites (Moore *et al.*, 1991). Invasion appeared to be associated with a chemical event that produced separation of epithelial junctions. This apparently permitted trophozoites to move into the intercellular space and to move laterally, burrowing under adjacent cells.

Once penetration has occurred, ongoing infection is dependent on availability of a suitable foodsource. Although environmental bacteria are its natural

foodstuff, bacterial co-infection is not a prerequisite for establishment of *Acanthamoeba* keratitis (Bacon *et al.*, 1993b). Within the epithelium, phagocytosis of cuboidal epithelial cells has been demonstrated in *in vitro* studies (Moore *et al.*, 1991). Intrastromal survival is assumed to be supported by consumption of keratocytes, as evidenced by keratocyte depletion in pathological specimens (Section 1.3.2).

The actual mechanism of invasion and cell destruction by *Acanthamoeba* is believed to be the result of phagocytosis and trogocytosis (piecemeal phagocytosis) in combination with the effects of amoebal secretions (Moore *et al.*, 1991). Taylor *et al.* (1995) suggest that contact-dependent lysis, similar to that observed with cytotoxic T cells and natural killer cells, may be important. Amoebostomes, sucker-like structures known to be involved in phagocytosis by *Naegleria* (Marciano-Cabral & John, 1983), have been described for *Acanthamoeba* (Diaz *et al.*, 1991; Pettit *et al.*, 1996). Alizadeh and co-workers (1994) have also suggested that *Acanthamoeba castellanii* produce cell death by stimulating the process of apoptosis in the target cell. The significance of finger-like projections on *Acanthamoeba* trophozoites, termed digipodia, is unresolved: it has been suggested that they are involved in initiating apoptosis (Pettit *et al.*, 1996).

With regard to amoebal secretions, He *et al* (1990) have demonstrated collagenolytic activity by secretions of *Acanthamoeba castellanii* both *in vitro*, and *in vivo*, in rat corneas. Collagenase alone was capable of producing a keratitis similar to that produced by the organisms themselves when inoculated into the stroma of Lewis rats. Data from Mitro *et al.* (1994) indicate that the secretions of *Acanthamoeba polyphaga* contain multiple serine and cysteine proteinases with non-specific collagenolytic activity and that metalloproteinases form an additional minor component. Neuraminidase activity has also been demonstrated for *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* (Pellegrin *et al.*, 1991). Elastase activity has been

demonstrated for both *Naegleria fowleri* and *Acanthamoeba culbertsoni* (Ferrante & Bates, 1988). In the latter study no difference was demonstrated between the levels of elastase in a highly pathogenic *Naegleria fowleri*, and those in the same organism that had lost pathogenicity following axenic maintenance.

In contrast, other studies of pathogenic and non-pathogenic strains of *Acanthamoeba* sp have demonstrated that, although the activity of a serine proteinase was similarly active in pathogenic and non-pathogenic strains, the activity of a cysteine proteinase was more marked in pathogenic strains (Hadas & Mazur, 1993b). Studies by the same authors also suggest a significant correlation between the activity of proteases, peroxidases and superoxide dismutases and the degree of virulence of the strains, as predetermined by their ability to infect mice (Hadas & Mazur, 1993a). In more detailed studies they have shown that the levels of peroxidase and proteinase activity showed an increase correlated with the increase in degree of virulence achieved by mouse passage of strains rendered nonvirulent by axenic maintenance (Mazur & Hadas, 1994). Furthermore, it was also claimed that strains that were previously non-virulent could be made virulent (Mazur & Hadas, 1994), using a modified method to evaluate invasiveness and virulence (Mazur & Jozwiak, 1993).

Thus *Acanthamoeba* secretions appear to be involved in pathogenicity. No similar studies have been performed with *Vahlkampfia* and *Hartmannella*. Analysis of secretions was outwith the scope of this project. In view of the cited reports, however, comparison of the ability of secretions of all three genera to produce cell damage is desirable. The relative contribution of other postulated mechanisms of cell damage also requires to be assessed. In spite of the inherent flaws, an *in vitro* model utilising corneal epithelial cells is appropriate in this context as it allows more detailed investigation of the different mechanisms.

Care is required, however, in the interpretation of the significance of ability to produce a cytopathic effect with regard to potential pathogenicity. While there is evidence to suggest that cytopathogenicity correlates with pathogenicity, the association is not absolute. For example, work by Cursons & Brown (1978) showed that various strains of the pathogenic *Naegleria fowleri* produced a cytopathic effect while various non-pathogenic strains of *Naegleria* did not. These authors therefore suggested that cytopathogenicity provides a rapid diagnostic indicator of potential pathogenicity. Another study involving 6 strains of *Naegleria* confirms this (De Jonckheere & van de Voorde, 1977), while suggesting that there is more of a continuum in virulence and that this is reflected in the degree of cytopathogenicity. De Jonckheere (1980) also suggests good correlation between potential neural pathogenicity of 19 *Acanthamoeba* species and cytopathogenicity. This agrees with work by van Klink *et al.* (1992) comparing the cytopathogenicity of an ocular and an environmental isolate of *Acanthamoeba castellanii.* They demonstrated that, although both were capable of producing a cytopathic effect, the relative cytotoxicity of the ocular isolate was greater. Visvesvara & Balamuth (1975) demonstrated that pathogenic *Acanthamoeba* sp Lilly strain not only differed from the non-pathogenic *Acanthamoeba castellanii* Singh strain as regards the ease of production of a cytopathic effect, but that the greater cytopathogenicity of the pathogenic strain was related to production of phospholipase. In contrast, however, Larkin *et al.* (1991) found that pathogenic and environmental *Acanthamoeba* produced a comparable degree of cytopathic effect. Similarly, in a study suggesting that only particular subsets of *Acanthamoeba* are virulent, the correlation between the ability to produce a cytopathic effect and the ability to establish keratitis in the rat model was reasonable (86%) but not absolute (Badenoch *et al.*, 1995). The significance of comparisons of cytopathogenicity between *Acanthamoeba, Vahlkampfia* and *Hartmannella* would therefore have to be interpreted cautiously with regard to potential pathogenicity *in vivo.* An *in vitro* model demonstrating invasion of human corneal tissue would provide important supplementary evidence in this respect.

Another confounding factor is that changes in culture conditions have been

shown to have profound effects on free-living amoebae. It has long been known that such changes may not only affect morphology (Sawyer, 1971; Stratford & Griffiths, 1978) but that axenisation may decrease cytopathic potential (Stevens & O'Dell, 1974; Cursons & Brown, 1978). More recent studies on *Naegleria* indicate just how far ranging these effects are. For example, two strains of *Naegleria fowleri,* LEE and LEEmpC1, were more similar in protein profiles and functional activity when both were grown in the same medium while differences in growth, proteins synthesised, cytopathogenicity, susceptibility to lysis by complement and rate of locomotion were noted when the same strain was grown in different media (Marciano-Cabral & Toney, 1994).

Furthermore, the effects of culture conditions are not limited only to the degree of cytopathogenicity: the mechanism of cytopathogenicity may also potentially be affected. For example, axenically cultured *Naegleria fowleri* (LEE) possess a distinctive surface structure termed an amoebastome which appears to be important in trogocytosis, as confirmed by electron microscope studies showing persisting areas of damage visible as craters in the target cells (Marciano-Cabral & John, 1983). The highly pathogenic mouse-passaged strain of *Naegleria fowleri* (LEEmp), however, possess relatively few such amoebastomes suggesting that trogocytosis is not the only mechanism responsible for effecting target cell injury (Marciano-Cabral, 1988). Indeed, they appear to primarily attack cells by lysis of the outer membrane, leaving the nuclear compartment relatively intact. Intimate contact between the cells is a prerequisite, with effector cell prongs being inserted into the target cell. More detailed studies (Marciano-Cabral *et al.*, 1990) indicate that the membrane fraction of LEEmp was more active than the soluble fraction in facilitating rubidium and chromium release, while conversely the soluble fraction of LEE was more active than the membrane fraction in facilitating rubidium release from radiolabelled target cells. The last authors postulate that the cytolytic factor(s) present in the axenically cultured strain may be used for internal digestion of ingested target cells while the cytolytic factors associated with the

membrane of mouse-passaged amoebae lyse target cells prior to ingestion of cellular debris.

In summary, therefore, the potential effects of culture conditions on both degree and mechanism of cytopathogenicity, would have to be born in mind when planning and interpreting investigations of the relative cytopathogenicity of *Acanthamoeba, Vahlkampfia* and *Hartmannella*.

1.4 Aims of Investigation

1.4.1 Diagnostic Techniques

1.4.1.1 Histology and immunodiagnosis

i) Comparison of the potential of 3 non-specific histological techniques, namely Grocott-Gomori methenamine silver, Alcian blue critical electrolyte concentration and calcofluor white, for detection of *Acanthamoeba* cysts and trophozoites in corneal tissue, with that of 3 techniques already in routine use (H&E, PAS, Giemsa).

ii) Assessment of the potential of 6 polyclonal antisera, raised against *Acanthamoeba*, for pan-amoeba and/or differential diagnosis of *Acanthamoeba*, *Vahlkampfia* and *Hartmannella* in corneal tissue.

iii) Investigation of putative cross-reactivity of one of the antisera (already in routine diagnostic use in the Pathology Department of Glasgow University) with respect to epithelial cells, keratocytes, monocytes, lymphocytes and polymorphonuclear cells.

1.4.1.2 Molecular biological techniques

i) Optimisation of the published PCR protocol for primers P1 and P2 with adaptation of the method for 'hot' PCR.

ii) Investigation of the sensitivity of PCR with primers P1 and P2 for detection of *Acanthamoeba*, including the potential to detect the organism in the

presence of corneal tissue and as both cyst and trophozoite forms.

iii) Assessment of the cross-reactivity of primers P1 and P2 with regard to detection of *Vahlkampfia* and *Hartmannella.*

iv) Adaptation of a standard ISH protocol for use with the labelled 126 bp probe complementary to the PCR product of primers P1 and P2.

v) Assessment of the method's potential for detection of cyst and trophozoite forms of *Acanthamoeba* in both corneal tissue and cytospin preparations.

vi) Investigation of the cross-reactivity of the method with respect to labelling of *Vahlkampfia* and *Hartmannella.*

1.4.2 Pathogenicity

1.4.2.1 Pathogenicity for corneal tissue

i) Development of a model method to demonstrate corneal invasion by *Acanthamoeba castellanii.*

ii) Description of the resulting pathology.

iii) Investigation of the ability of *Vahlkampfia* and *Hartmannella* to demonstrate invasion of corneal tissue.

1.4.2.2 Pathogenicity for corneal cells

i) Description of the cytopathic effect produced with time on cultured keratocytes exposed to various concentrations of *Acanthamoeba castellanii.*

ii) Comparison of the relative cytopathogenicity of *Acanthamoeba, Vahlkampfia* and *Hartmannella* for cultured keratocytes.

iii) Comparison of the relative susceptibility of cultured epithelial cells and keratocytes to damage mediated by *Acanthamoeba castellanii.*

1.4.2.3 Mechanism of cytopathogenicity

i) Study of the cytopathic effect on keratocytes resulting with each of the 3 genera of amoebae using time lapse video microscopy and SEM, with emphasis on elucidation of the mechanisms of cell destruction.

ii) Assessment, for all 3 genera, of the role of secretions, as opposed to physical methods, in production of the observed cytopathic effect.

iii) Investigation of the postulated involvement of apoptosis in cell death mediated by *Acanthamoeba castellanii*.

TABLE 2.1

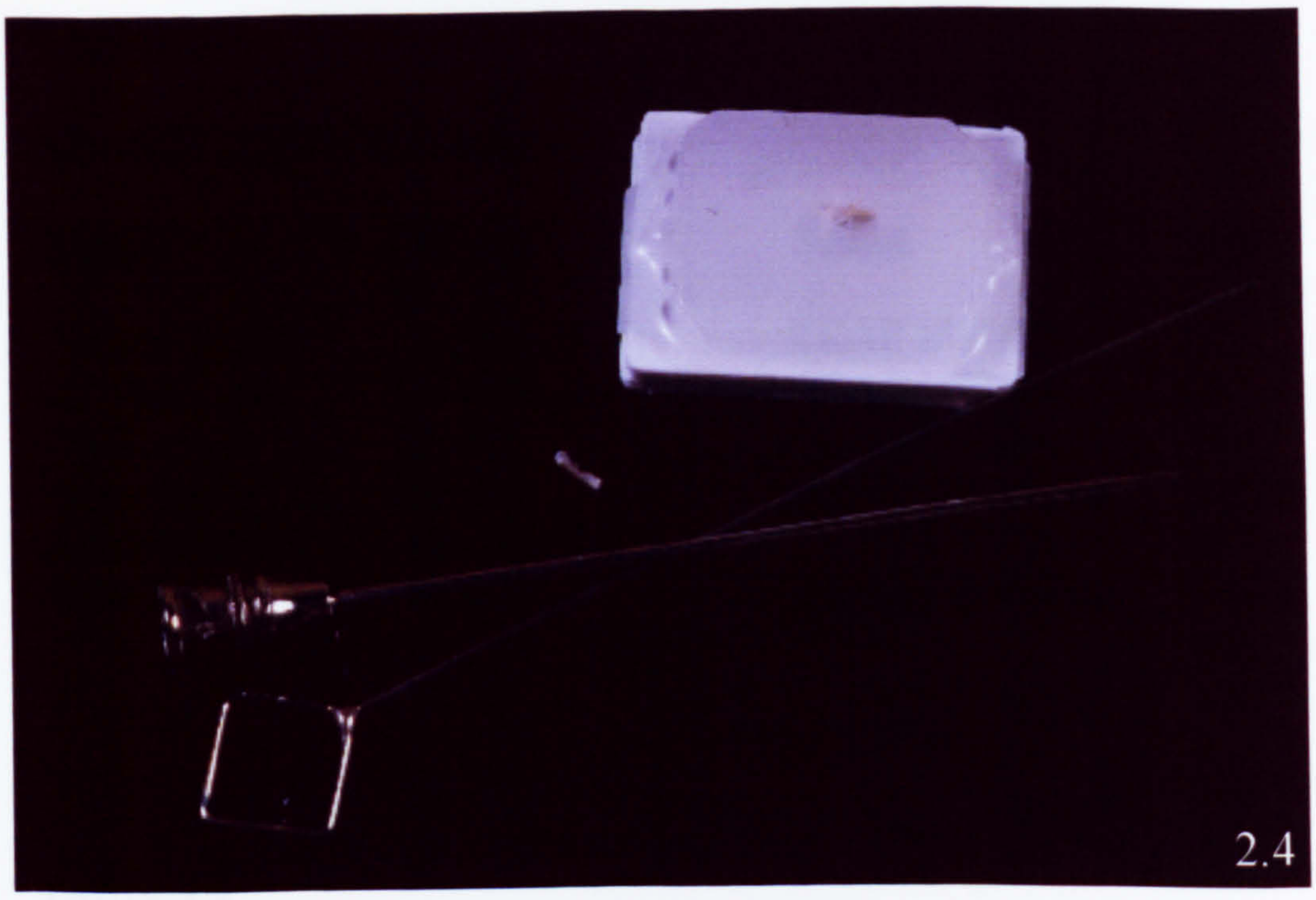
Amoebae Species and Sources

Genera	Species	Source	Code
Acanthamoeba	*castellani*	CCAP 1501/2D	AC
Acanthamoeba	*polyphaga*	CCAP 1501/3G	AP
Vahlkampfia	*avara*	CCAP 1534/7A	VA
Vahlkampfia	*? sp*	Patient GM-cornea	V-EYE
Hartmannella	*vermiformis*	CCAP 1588/1B	HV
Hartmannella	*? sp*	Patient GM-cornea	H-EYE

TABLE 2.2

Anti-amoeba Polyclonal Antisera

Immunogen	Source	Code
Acanthamoeba Neff	David Warhurst	DW anti-*Acanthamoeba* Neff
Acanthamoeba HN3	David Warhurst	DW anti-*Acanthamoeba* HN3
Acanthamoeba sp	Simon Kilvington	SK anti-*Acanthamoeba*
Acanthamoeba sp	Glasgow Royal Infirmary	GRI anti-*Acanthamoeba*
Vahlkampfia sp	Glasgow Royal Infirmary	GRI anti-*Vahlkampfia*
Hartmannella sp	Glasgow Royal Infirmary	GRI anti-*Hartmannella*



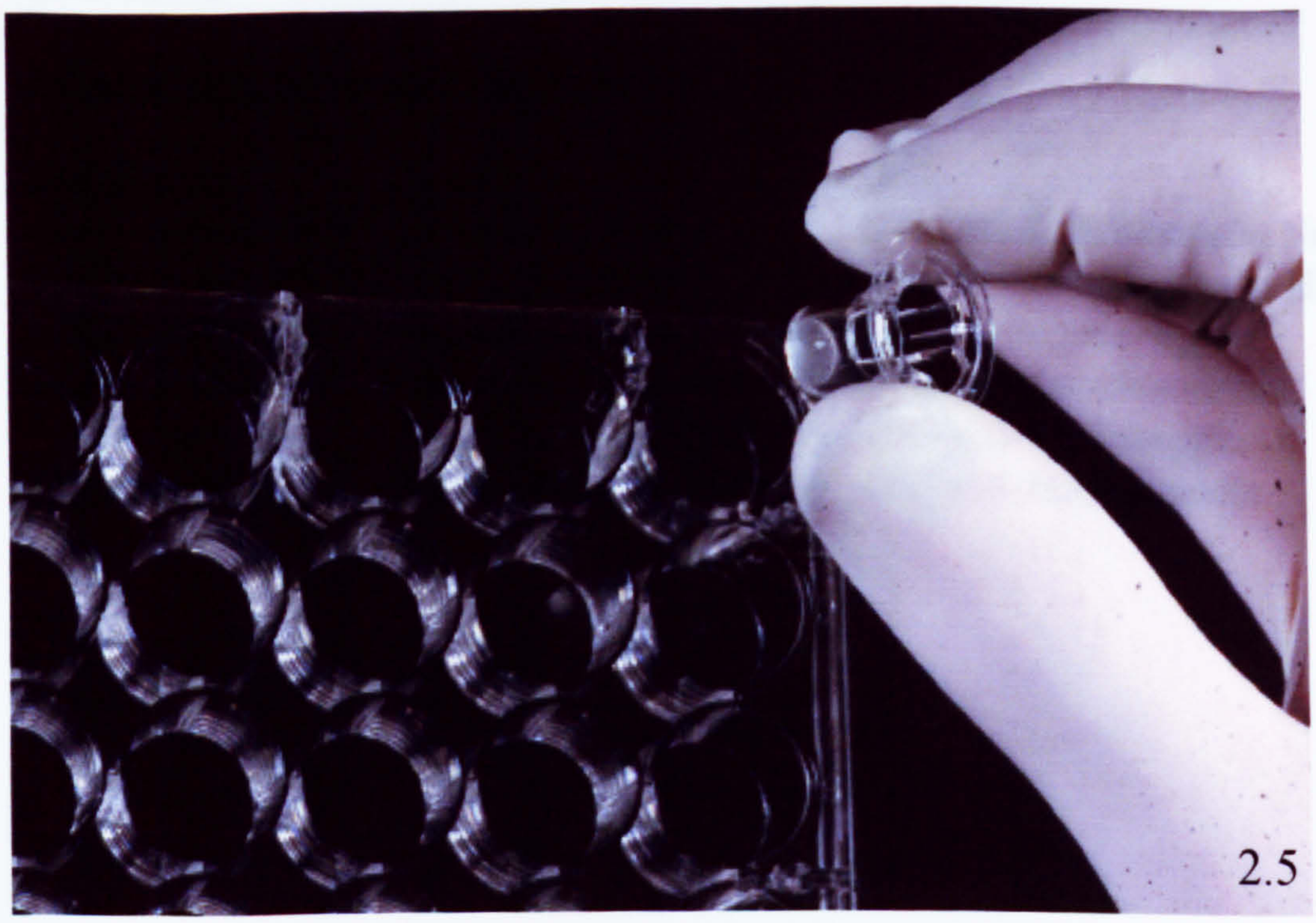
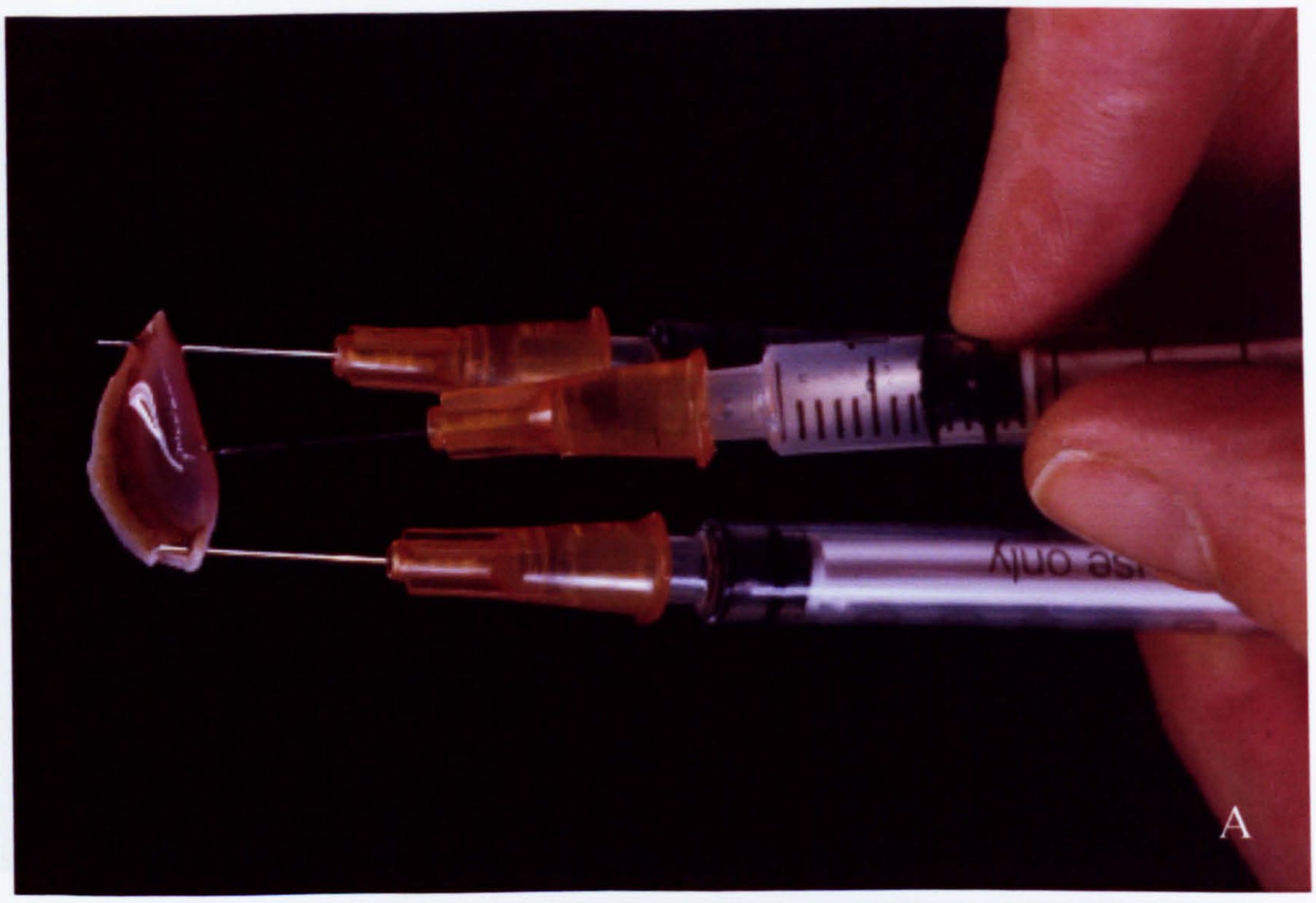


Figure 2.4 Core biopsy method
Core biopsy instrument and core of tissue removed from paraffin-embedded tissue. This is then re-embedded for sectioning, thus preserving precious pathological specimens.

Fig 2.5 Transwell apparatus
Twenty four well plate with separate mesh-bottomed insert. This set-up allowed investigation of the effects produced by incubation of cultured cells in the presence of live amoebae, but in the absence of physical contact.



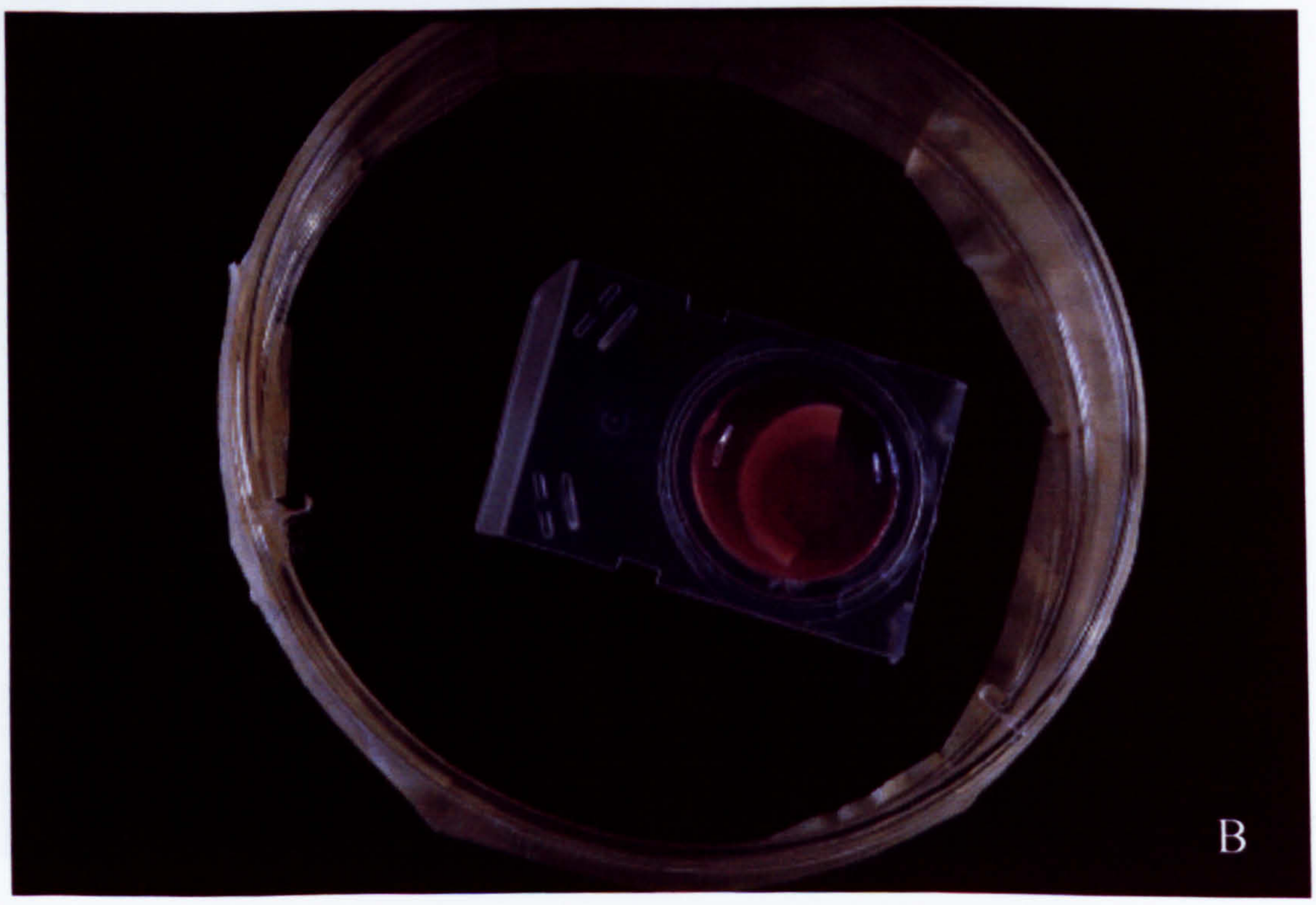
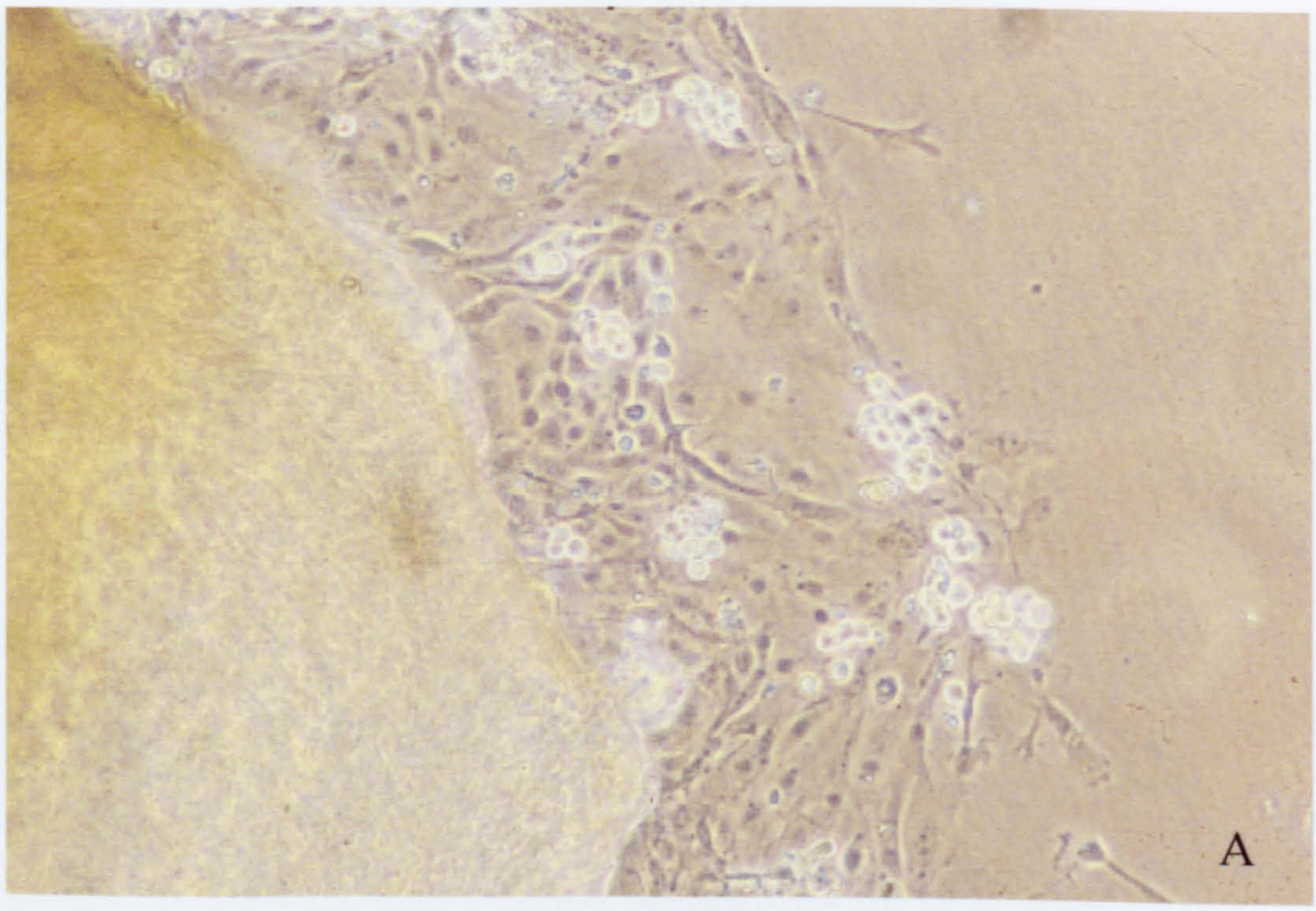
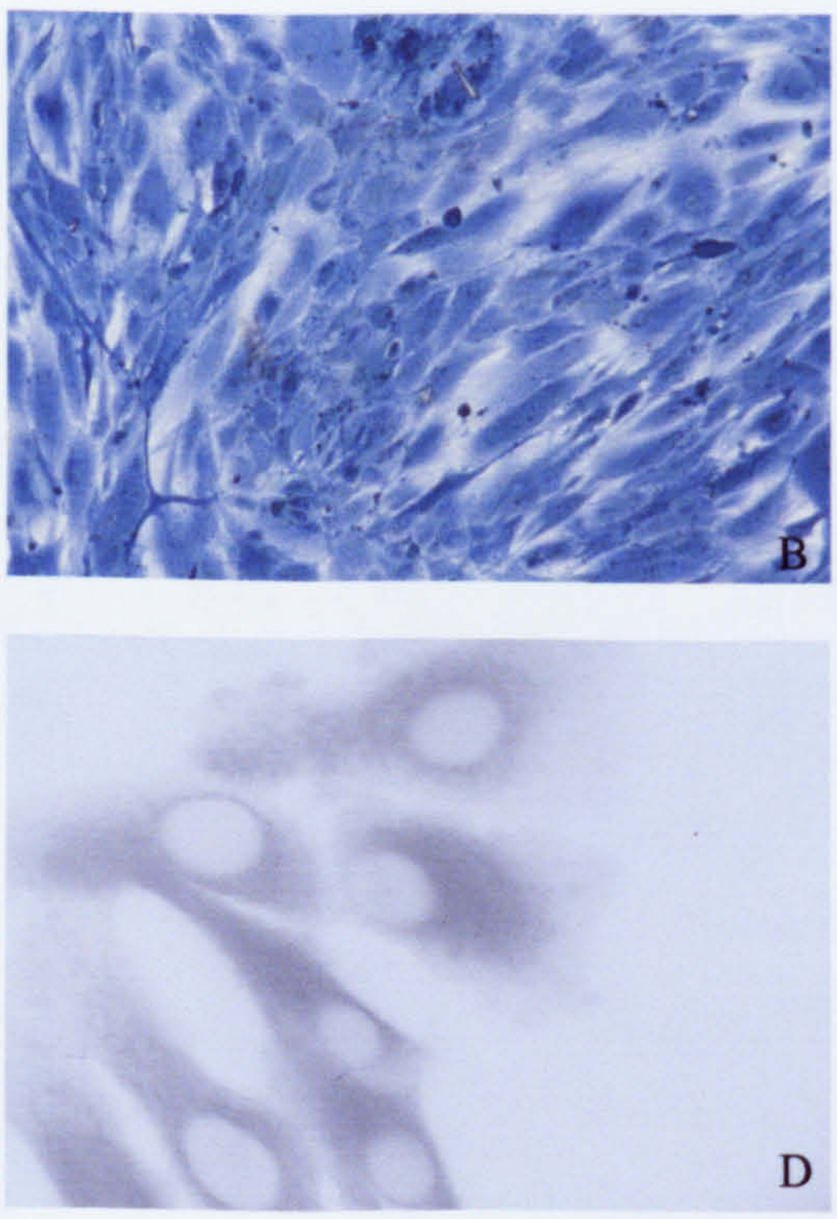


Fig 2.3 Methods of corneal tissue preparation.

A) Known amoebae trophozoites and/or cysts were inoculated into corneal tissue with an insulin syringe as demonstrated. The tissue thus prepared was used for comparison of the diagnostic ability of histological stains, immunolabelling and *in situ* hybridisation. The behaviour of amoebae within the stroma, and the resulting pathology, were also investigated.

B) The 'definitive' method of assessing the ability of the 3 different genera to invade, involved incubation of a whole human cornea, epithelium downwards, on top of a daily wear contact lens which was supported in its own holder. Although, for demonstration purposes, a half cornea is shown in this picture, in practise a whole cornea was used.





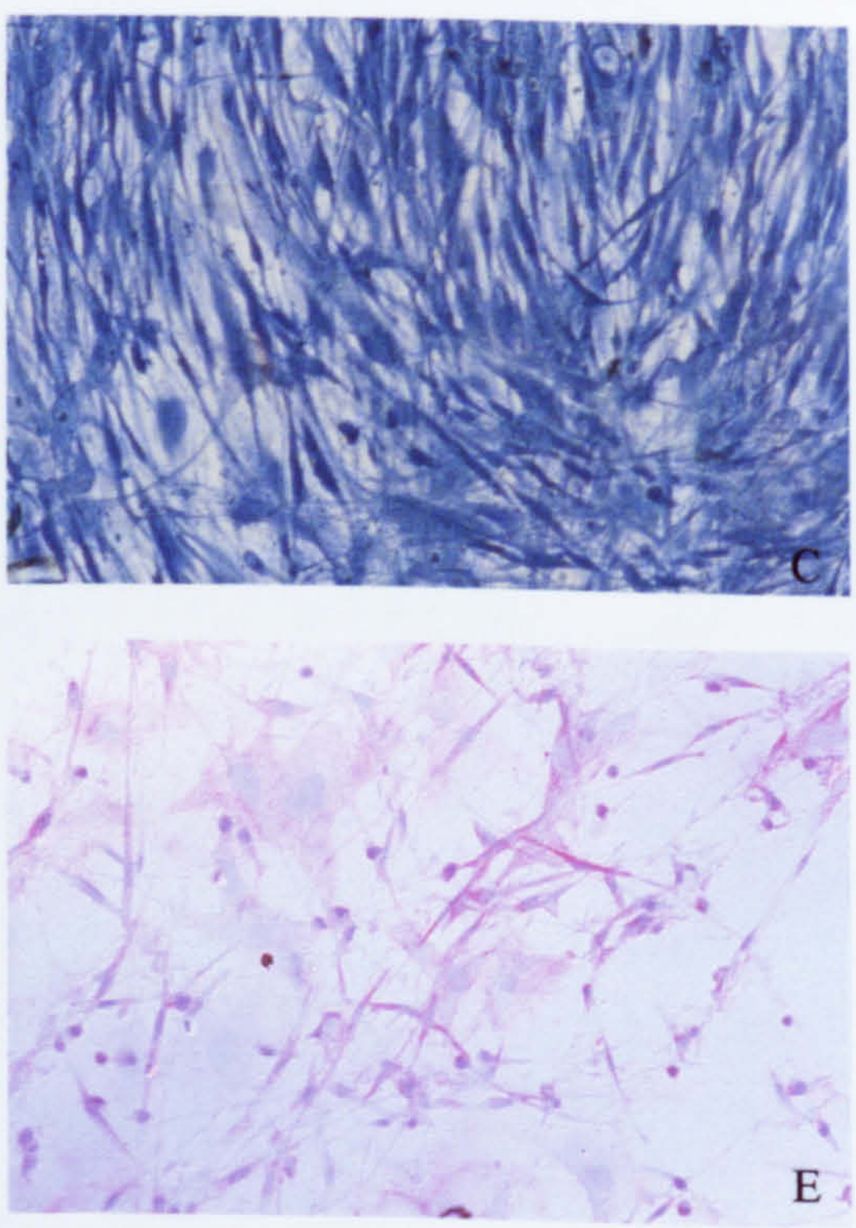
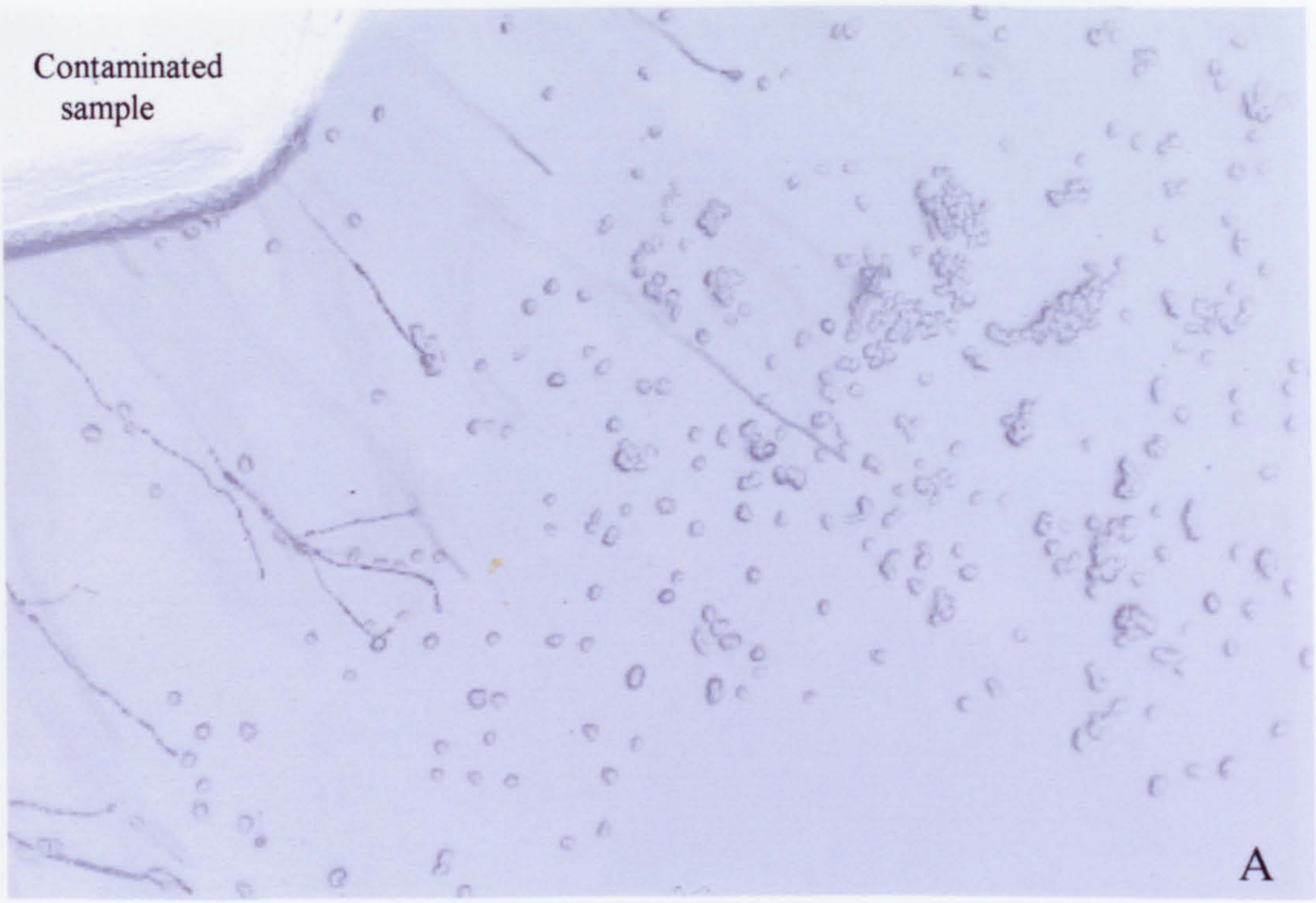


Fig 2.2 Corneal epithelial cells and keratocytes

A) Primary culture of epithelial cells demonstrating migration from surface of cornea prior to appearance of keratocytes. (x160)
B) Coomassie blue-stained preparation of cultured epithelial cells demonstrating typical 'crazy paving' appearance of monolayer, with a continuous sheet of epithelial cells joined by tight junctions. (x200)
C) Coomassie blue-stained preparation of keratocytes demonstrating typical fibroblastic appearance of cells and random arrangement in 'monolayer'. (x200)
D) Epithelial cells labelled with anti-pancytokeratin antibody (black and white photograph of indirect immunofluorescence - x400).
E) Keratocytes labelled with anti-fibroblast antibody (alkaline phospatase method: mag x90).



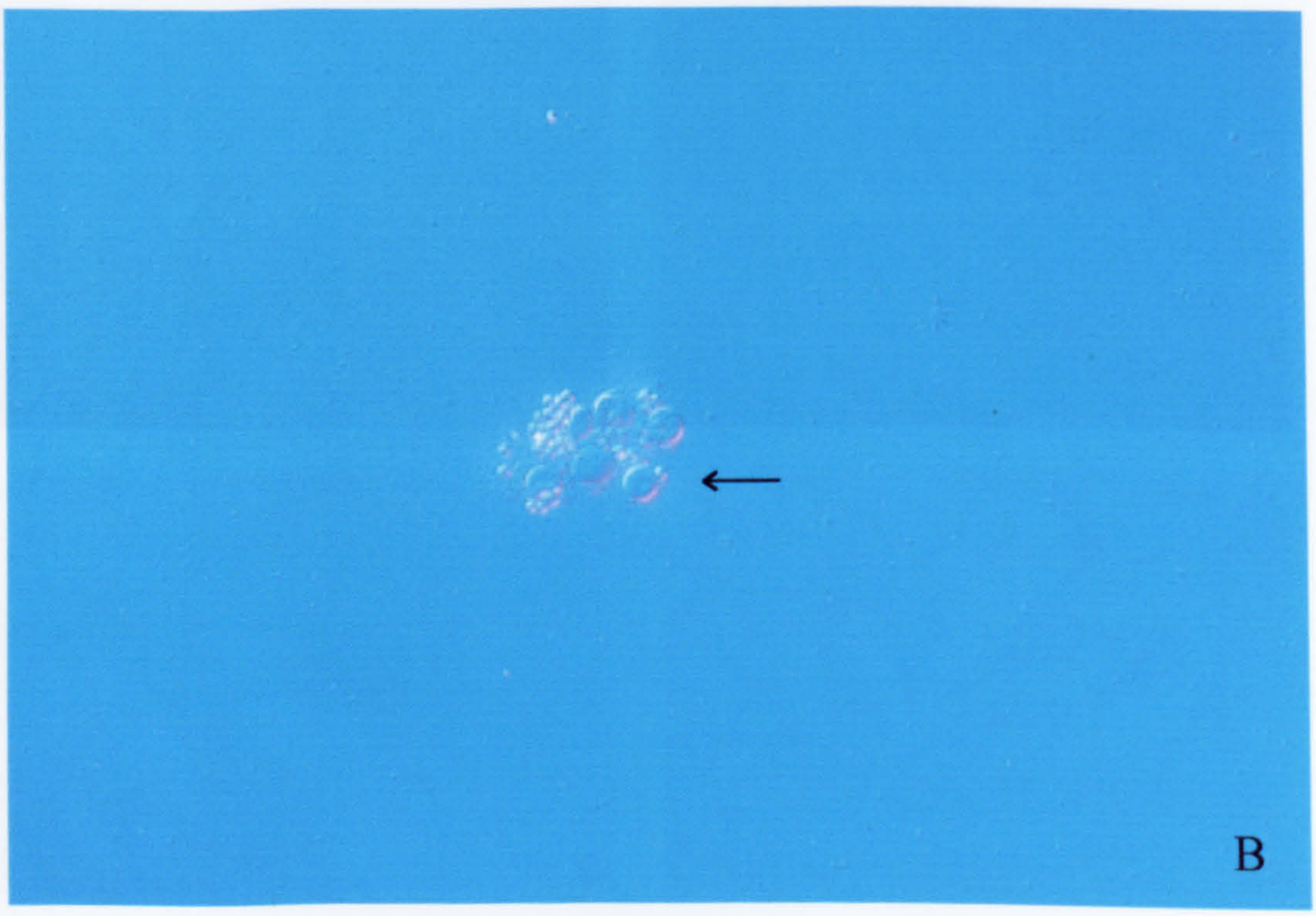


Fig 2.1 Purification of contaminated cultures.

A) Trophozoites migrating clear of the more slowly invasive hyphae of a fungal contaminant.
(Phase contrast microscopy: mag x85)

B) A group of V-EYE cysts, contaminated with yeasts. Note how the yeasts appear to be associated with the mucoid coat of the cysts (arrow).
(Interference contrast microscopy: mag x340)

2. MATERIALS AND METHODS

2.1 Preparations

2.1.1 Amoebal Culture

2.1.1.1 Species and sources

The different species and strains of amoebae used in this study are illustrated in Fig 1.1. They were obtained from various sources as detailed in Table 2.1. All amoebae were handled using aseptic technique at all times, in an Intermed Microflow Pathfinder class II laminar flow cabinet whenever practical, with disposal of waste according to hospital safety regulations.

2.1.1.2 Monoxenic growth on agar

All species of amoebae were obtained, and initially maintained, as monoxenic cultures on petri dishes of 1% non-nutrient agar (Appendix A) seeded with heat-killed *Klebsiella pneumoniae* (Appendix A). This was supplemented with 2-3 ml of amoebal saline (Appendix A) or normal saline prior to sealing with tape to maintain a moist environment. An incubation temperature of 34 ^{0}C was chosen in order to closer mimic conditions in the cornea *in vivo* (Efron *et al.*, 1989). This was considered to yield more prolific growth than either 37 ^{0}C, or 40 ^{0}C; the ability to grow at this latter temperature has been suggested as being associated with likely virulence (De Jonkheere, 1980; Griffin, 1972).

2.1.1.3 Purification: mixed cultures

The specimens of *Vahlkampfia* and *Hartmannella* isolated from the corneal biopsy of patient GM (Aitken *et al.*, 1996) were received as a mixed culture. Establishment of pure cultures of these particular *Vahlkampfia* and *Hartmannella* strains, termed V-EYE and H-EYE for convenience, took several months; the process was complicated by the presence of a sticky mucoid layer surrounding the *Vahlkampfia* (Fig 2.1B), to which other amoebae tended to adhere. To achieve this end, the migration method described by Neff

(1957) was adapted as follows. Basically a sample of amoebae was inoculated onto the middle of a non-nutrient agar plate while seeding only the periphery of the plate with heat-killed bacteria. Using a Leitz DIAVERT inverted phase contrast microscope, individual amoebae of a single strain, which had fortuitously migrated relatively clear of the rest, were identified and plated onto fresh agar plates using a scalpel blade. This process was repeated until pure cultures of the 2 separate genera had been established.

2.1.1.4 Purification: contaminated cultures

Prior to axenisation the amoebae required to be rendered free from any contamination which would also thrive in the axenic medium. Fungal contamination was eliminated by means of the adapted migration method described above (Section 2.1.1.3) while an acid wash technique (Connor *et al.*, 1993) was utilised to decrease bacterial contamination. Using the former technique the amoebae migrated clear of the (more slowly invading) fungal hyphae (Fig 2.1A). Attrition of egested bacteria is also achieved by this method (Neff, 1958). For the acid wash technique the amoebae were first protected by causing them to encyst; this was achieved by making the environment unfavourable (i.e. by starvation). Following detachment from the agar surface by pipetting with normal saline and/or scraping with a cell scraper (Costar Ltd), they were pelleted by centrifugation at 1200 g for 5 min. They were then exposed to a 3% solution of concentrated HCl for 30-60 min. After a further centrifugation at 1200 g for 5 min to pellet, they were washed with 10 ml of normal saline, repelleted, washed with a second 10 ml of normal saline and repelleted.

2.1.1.5 Axenic culture and monoxenic growth in suspension

Following the final wash the amoebal cysts were resuspended in 2-3 ml of heat-killed bacteria, and transferred to 25 cm^2 tissue culture flasks (Costar Ltd). After 1-2 days, when the amoebae had excysted, 2-3 ml of axenic growth medium were added. The medium was changed at regular intervals by gently

pipetting off the surface layer, taking care not to disturb the trophozoites adhering to the bottom of the flask. In this fashion the heat-killed bacteria were progressively diluted out as the amoebae became accustomed to the axenic growth medium as a food source.

Attempts to establish true axenic growth were both fraught with difficulties and extremely time-consuming. Repeated attempts, each lasting several months, were made over a two year period. In spite of the acid wash technique, contamination was still a problem; this was partly because the amoebae can harbour live intracellular bacteria that are subsequently egested, and partly because of relative insensitivity of fungal spores and yeasts to the acid wash technique. Axenisation of the isolate of *Vahlkampfia* from patient GM was also complicated by the presence of a mucoid layer that appeared to protect adherent organisms (Fig 2.1B). Furthermore, the growth of these small free-living amoebae often appears erratic, with sudden failure to thrive when apparently identical conditions previously supported growth. Frequently the amoebae would appear to adapt to a particular axenic growth medium only to subsequently replicate poorly, become stunted in appearance and finally peter out altogether.

For each strain attempts were made, at least twice in each case, to establish axenic growth in each of 8 different axenic media. The media utilised were Proteose peptone-Glucose Medium, Proteose peptone-Yeast extract-Glucose-Serum Medium, Modified Chang's Medium, Neff's Optimal Growth Medium, Proteose peptone-Yeast extract-Glucose Medium (Visvesvara, 1995), *Balamuthia* Medium (Schuster & Visvesvara, 1996), PYNFH Medium and FKMx4 Medium (all listed in Appendix A). By this process *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* were adapted to growth in Proteose peptone-Yeast extract-Glucose (PYG) medium but axenic cultures of V-EYE and H-EYE were never successfully established. It has been suggested that there is a correlation between ease of axenisation and cytopathic potential (De Jonckheere, 1980).

Due to the failure to axenise cultures of V-EYE and H-EYE, monoxenic cultures were adapted for growth in 25 cm^2 culture flasks, fed by a suspension of heat-killed *Klebsiella pneumoniae*. Although not as desirable as use of axenic cultures for pathogenicity studies, this method did at least permit growth and relatively efficient harvesting of large numbers of organisms without agar debris.

2.1.1.6 Harvesting

When amoebae were required, they were harvested as follows, in the logarithmic phase of growth if destined for pathogenicity experiments (Chapter 4). After leaving at 4 ^0C for 10-15 min, the amoebae were detached from the plastic tissue culture flask using a cell scraper and centrifuged at 1200 g for 5 min to pellet. They were then washed with 10 ml of normal saline, repelleted, washed with a second 10 ml of normal saline, counted in an Improved Neubauer Chamber, and repelleted before suspending at the required concentration in appropriate medium.

2.1.2 Specimens for Assessment of Diagnostic Methods

2.1.2.1 Cytospin preparations

Cytospin preparations of *Acanthamoeba castellanii* were prepared for studies of the potential of calcofluor white to detect both trophozoite and cyst forms of the organism. Cytospin preparations of trophozoite and cyst forms of each of the various amoebae (Table 2.1), and of two unidentified *Acanthamoeba* sp isolated from patient corneal biopsies, were also prepared for studies to assess the potential for differential diagnosis of the three genera by immunolabelling (see Sections 2.2.1.2 & 3.2.1.2 for details of the method and the experiments performed respectively) and also by ISH (see Sections 2.2.3 & 3.2.2.2 for details of the method and the experiments performed respectively). Cytospin preparations of polymorphs, lymphocytes and monocytes which had been pre-

exposed to *Acanthamoeba castellanii* were also utilised to investigate potential antibody cross-reactivity (see Appendix C for separation method and Section 3.2.1.2 for co-incubation methods). In each case three drops of a suspension of 1 x 10^6 cells/ml were spun onto individual APES-coated slides (Appendix C) at 450 rpm for 5 min, then fixed in neutral buffered formalin (Genta Medical) or in acetone if intended for immunofluorescence studies.

2.1.2.2 Corneal specimens

Human corneal tissue was inoculated with known amoebae to provide test tissue for investigation of the differential diagnostic ability of immunolabelling (Sections 2.2.1.2 & 3.2.1.2) and ISH (Sections 2.2.3 & 3.2.2.2).

The corneas were obtained from the eyebank, having been rejected for grafting purposes due to low endothelial counts (Eyebank, Bristol Eye Hospital, Lower Maudlin Street, Bristol BS1 2LX). The corneas were both provided and stored at 4 ^{0}C in hypotonic organ culture medium (Pels & Schuchard, 1983).

On receipt there was little or no epithelium present and keratocytes were variable in both number and appearance (personal observations). It is unknown whether the apparent keratocyte depletion was related to lack of epithelium (Campos *et al.*, 1994; Wilson *et al.*, 1996) or to extended organ culture. All histological studies were performed within 4-8 weeks of harvesting.

For inoculation experiments, the use of hypotonic solutions induced moderate swelling and softening of the corneal stroma and thus facilitated introduction of amoebae into this tough, collagenous tissue. The procedure was performed aseptically and required only a half cornea, but complete with scleral rim. Some practice was required with this rather fiddly technique, details of which are given in the following paragraph. A similar inoculation method was utilised for pathogenicity studies, and is described in full in Sections 2.1.3.1, 2.3.1.2 and 4.2.1.2. In total, over 50 corneal injections were performed.

Inoculations were performed for all 6 species, representing the 3 different genera, as listed in Table 2.1. Using the procedure described in Section 2.1.1.6, the organisms were harvested in large numbers. As far as possible, a mixture of

trophozoites and cysts forms (50:50) was utilised for tissue intended for diagnostic studies. After resuspending in 0.1-0.2 ml of normal saline, a one ml/1000 U disposable insulin syringe (0.33 mm x 13 mm) was used to introduce the amoebae into the tissue; it was inserted at right angles to the cut edge of the half cornea, advanced as far as the limbus and then slowly withdrawn while the amoebae were expelled from the syringe using minimal force (Fig 2.3A).

Post-instillation of the organisms the corneal tissue was incubated at 34 ^{0}C for 2 hr in hypertonic saline (1.8% NaCl), or overnight in organ culture medium made isotonic by supplementing with 5% Dextran T500 (Pels & Schuchard, 1983). The latter is the medium into which donor corneas are transferred prior to grafting. Preliminary experiments had demonstrated that these 2 alternative treatments acted to restore the transparency and hence the tissue architecture of the swollen cornea. Tissue was then immersion-fixed in neutral buffered formalin, embedded in paraffin wax and 5 μm sections containing known amoebae in corneal tissue, cut for investigation of the diagnostic potential of immunolabelling and ISH. The relevant methods are described in Sections 2.2.1.2 & 2.2.3 respectively, while the experiments performed are detailed in Sections 3.2.1.2 & 3.2.2.2 respectively.

2.1.2.3 DNA extraction

In order to obtain DNA samples for PCR, monoxenically cultured amoebae, harvested from agar plates as previously described for flasks (Section 2.1.1.6), were incubated overnight at 56 ^{0}C in a sealed Eppendorf in 100 μl of proteinase K digest solution (Appendix D). The PCR method is detailed in Section 2.2.2.2 while the experiments themselves are described in Section 3.2.2.1.

2.1.3 Corneal cells and Tissue for Pathogenicity Studies

2.1.3.1 Source

Preliminary experiments investigating the ability of the amoebae to invade corneal tissue, and the resulting pathology, utilised fresh rabbit corneas available on a regular basis from rabbits culled for an unrelated study that did not involve the eyes. Later experiments utilised eyebank corneas. As already described, these had been rejected for keratoplasty due to low endothelial counts and were received in hypotonic eyebank medium (Section 2.1.2.2). For the purpose of invasion, however, they were incubated overnight in isotonic eyebank medium (i.e. supplemented with 5% Dextran T500) to restore tissue architecture prior to co-incubation with amoebae.

Before invasion had been successfully demonstrated in the above experiments, eyebank corneas were also inoculated with *Acanthamoeba castellanii* in order to investigate their behaviour within corneal tissue, and the resulting pathology. The method utilised was similar to that used to prepare tissue for testing diagnostic methods (Section 2.1.2.2); it is described in Section 2.3.1.2, while the experiments themselves are detailed in Section 4.2.1.2.

Human corneal epithelial cells and keratocytes were obtained by primary culture from corneal buttons removed at grafting from patients with keratoconus and from corneal buttons recovered from eyes enucleated for treatment of choroidal melanoma. Over a dozen different attempts were required to amass sufficient numbers of cells. This was mainly due to difficulties encountered with primary isolation of epithelial cells: they had more demanding growth requirements than keratocytes and were readily lost from culture if contact with the substratum was not established within a limited time frame of a few days.

Using a sterile technique, the corneas were dissected, as described in Stocker *et*

al. (1958), so as to remove the endothelium and separate the epithelium from the bulk of the corneal stroma. Small blocks (<1 mm^3) of either epithelium plus a small amount of stroma, or stroma alone, were then placed in individual wells of 24 well plates with 1-2 drops of Endothelial Cell Growth Medium (Appendix B) supplemented with 20% foetal calf serum (EGM 20%) to maintain moistness on incubation in a humidified incubator at 37 ^{0}C in 5% CO_2 overnight. The following day, when the individual blocks had adhered to the bottom of the plastic, 2-3 ml of EGM 20% were added.

2.1.3.2 Separation of corneal cells

Using an inverted phase microscope, the plates were then observed on a daily basis for migration of epithelial cells from the surface of the cornea onto the well bottom (Fig 2.2A); this process occurred after approximately 3-5 days. When the epithelial cells had formed an adherent sheet of a few millimetres, the block of tissue was removed prior to the emergence of keratocyte migration and growth at 7-10 days. By this method primary cultures of corneal epithelial cells were obtained from those blocks of cornea consisting of epithelium plus some stroma: similarly primary keratocyte cultures were established from those blocks of cornea consisting of stroma alone. Epithelial cells formed a monolayer with a typical crazy paving appearance while keratocytes, which are fibroblastic type cells, did not (Figs 2.2B & C). The origin of the cell types was further confirmed by labelling by indirect immunofluorescence with mouse anti-human pancytokeratin antibody (Sigma) for epithelial cells and by the indirect alkaline phosphatase technique with anti-fibroblast antibody (DAKO) for keratocytes as described in Appendix B (Fig 2.2D & E). The negative control in each case consisted of the opposite cell type.

2.1.3.3 Growth and maintenance

Once the 2 types of cell cultures had become sufficiently established they were transferred to 25 cm^2 tissue culture flasks with CO_2 vents, then similar 75 cm^2 flasks, for maintenance culture with serial passage, splitting 1:3 on confluence.

At each transfer the cells were detached from the plastic flask bottom by trypsinisation. This involved washing twice with HEPES-saline (Appendix B) to remove serum, washing with trypsin-versene (Appendix B) and then, after pouring off most of the trypsin-versene, incubating at 37 ^{0}C for approximately 5 min, until the cells were seen to detach when they were resuspended in fresh EGM 20%. As sufficient numbers of cells were grown, backup aliquots of cells in 75 cm^2 flasks were trypsinised, suspended in 1 ml of freeze mix (Appendix B) and stored frozen at -70 ^{0}C. When the frozen cultures were required they were thawed at 37 ^{0}C, added to 10 ml of EGM 20%, pelleted by centrifugation at 1200 revs for 10 min, washed again in a further 10 ml of the same medium, repelleted, then resuspended in 5 ml of EGM 20% for plating out into a 25 cm^2 culture flask. The cultured cells utilised in the experiments were all less than a year old, including frozen storage time, and had all been passaged a total of 6-9 times. Although cells were isolated from several different corneas, all cells used in any one experiment were from the same source. The methods involved are described in Section 2.3.2 while the experiments themselves are detailed in Section 4.2.2.

2.2 Diagnostic Methodologies

2.2.1 Histology and Immunodiagnosis

2.2.1.1 Histology

H&E, Giemsa, PAS, Grocott-Gomori's methenamine silver and Alcian blue critical electrolyte stains were performed according to Bancroft & Stevens (1996). The method for calcofluor white is detailed in Appendix C. The results of this technique and the other fluorescent methods were visualised on a Leitz Orthoplan 250UV microscope. Details of the experiments performed to test the ability of these non-specific stains to identify amoebal trophozoites and cysts in corneal tissue are listed in Section 3.2.1.1.

2.2.1.2 Immunolabelling

Six different anti-amoebal antisera were available for study (Table 2.2). Three of these antisera were in routine diagnostic use and were putatively specific for *Acanthamoeba*: DW anti-*Acanthamoeba* Neff, DW anti-*Acanthamoeba* HN3 and SK anti-*Acanthamoeba* obtained courtesy of Dr David Warhurst (Amoebiasis Unit, Hospital for Tropical Disease, London NW1 0PE) and Dr Simon Kilvington, (Public Health Laboratory, Combe Park, Bath BA1 3NG) respectively. These were compared with three locally produced antisera from Glasgow Royal Infirmary, termed GRI anti-*Acanthamoeba,* GRI anti-*Vahlkampfia* and GRI anti-*Hartmannella*. The GRI antisera had been prepared using cultures from the Culture Collection of Algae and Protozoa (CCAP, Freshwater Biological Association, The Ferry House, Ambleside, Cumbria LA22 0LP) which were inoculated into the rabbit as a mixed suspension of formaldehyde-killed trophozoites and cysts in incomplete Freud's adjuvant, followed by an inoculation of live protozoa (10^5/ml) without adjuvant 24 days later and exsanguination a further 4 weeks later. All the antibodies tested were polyclonal antisera raised in rabbits against the named genera of amoeba. Immunolabelling using indirect immunofluorescence, indirect alkaline phosphatase and ABC-Peroxidase techniques were carried out according to standard methods (Appendix C). The experiments performed to test the specificity and cross-reactivity of these antisera are described in Section 3.2.1.2.

2.2.2 Polymerase chain reaction (PCR)

2.2.2.1 Primers and probes

The following primers and probe, selected from the literature (Lai *et al.*, 1994), were manufactured on a 1.0 μmole scale on a 381A DNA synthesiser from Applied Biosystems by Dr VB Math, Institute of Biomedical and Life Sciences, Glasgow University:

19 bp primer P1: 5' GGA GCT CCC ACG GGA GGC C 3'

22 bp primer P2: 5' TGG ACC GCG TGA GGC TGC GGC T 3'

126 bp probe ArDNA-a: 5' GGA TCC TGG ACC GCG TGA GGC TGC GGC TGG GCT GCG CTG TGA CTA CTG CCG TGC GGC GTC AAA ACC GTG CGG TGG GAA AGT GGT GCC CTG GCT TTG GCC GGT TTG CGC GGG CCT CCC GTG GGA GCT 3'

The trityl group was deprotected as described in Technical Bulletin 041 in order to release the oligonucleotide, which was then purified on an oligopurification cartridge (Cruachem Ltd) according to the instructions in Technical Bulletin 043. The concentration of each purified oligonucleotide was estimated by spectrophotometry and adjusted to give an OD_{260} of 7, prior to lyophilisation for storage at -20 ^{0}C. The experimental methods related to PCR that were performed with these primers and probes are detailed below (Sections 2.2.2.2 - 2.2.2.5) while ISH utilising the 126-bp probe is described in Section 2.2.3. Details of the experiments themselves are listed in Sections 3.2.2.1. & 3.2.2.2. for PCR and ISH respectively.

2.2.2.2 Amplification

Using DNA samples extracted by proteinase K digestion (Section 2.1.2.3), both standard 'cold' PCR and 'hot' PCR were performed on a Hybaid Thermal Reactor. The protocol for 'cold' PCR is detailed in Appendix D. 'Hot' PCR is a much more sensitive technique capable of detecting one base pair differences in the amplicon. It was performed as for 'cold' PCR except that the dNTP mix contains only 10% of the amount of dCTP in the 'cold' PCR mix. This was supplemented by adding 0.1 µci (0.1 µl) of 5' P32 dCTP (Amersham LIFE SCIENCE) per 25 µl reaction volume to the master mix. Post-PCR, the products were run on a gel. In the case of 'cold' PCR, a standard 2% agarose gel containing ethidium bromide was used (see Appendix D), In contrast, following 'hot' PCR the products were loaded as 2 µl of sample in 2 µl of loading dye 2, along with a sequencing ladder, on a polyacrylamide gel (prepared and run as described for 'sequencing gel' in Appendix D).

Precautions were also taken to minimise the chance of contamination. Sample

preparation, PCR amplification and product visualisation were all performed in different areas. Nonpyogenic water and frequently changed aliquots of reagents were used with plugged pipette tips, dedicated pipettes (positive displacement where possible) and sterile Eppendorfs. Mineral oil was autoclaved before use. Multiple negative controls were also employed.

2.2.2.3 Southern blotting

Southern blotting was performed on 'cold' PCR products prepared and run in a 2% agarose gel containing ethidium bromide to test if the technique had yielded the expected amplicon. The gel was first denatured in SB denaturing solution (Appendix D) for 30 min, to get single stranded DNA followed by neutralisation (<pH 9) in SB neutralisation solution (Appendix D) for 30 min.

The DNA was blotted from the gel by capillary transfer as follows. Filter paper was placed over an upturned cassette in a blotting tank filled with 20X SSC buffer (Appendix D). The ends of the filter paper must be in contact with the buffer. The gel was placed (right way up) on the filter paper with Hybond N (Amersham LIFE SCIENCE) on top of the gel and a gasket on top of the Hybond N. A smaller piece of filter paper (which must not dip into the buffer) was added, with layers of folded up, absorbent paper on top, all weighed down with a 1kg weight. This was left overnight to allow transfer of the DNA from the gel to the Hybond N, which was allowed to dry and fixed by baking at 80 ^{0}C for 2 hr.

A standard southern blotting hybridisation protocol (Appendix D) was then applied to the blot to test the transferred PCR product with the complementary 126-bp probe ArDNA-a. In order to allow visualisation of the PCR product-probe complex, if present, the probe had been previously labelled at the 3' end with digoxigenin-11-dUTP/dATP using the Boehringer Mannheim DIG Oligonucleotide Tailing Kit (Appendix D).

2.2.2.4 Cloning

Cloning of DNA was carried out as described below, using the pMOS*Blue* T-vector Kit RPN 1719 (Amersham LIFE SCIENCE). Prior to ligation into the vector, the PCR product to be sequenced was purified by running on an agarose gel with ethidium bromide and excising the relevant band that was then passed through a SPIN-X column (Costar Ltd). The quantity of DNA present was estimated by spectrophotometry (Shimadzu UV-160A) using the following formulae: uM = OD_{260} x 100/No of bases and 1 M = 500 Da x No of bases g/L.

For optimal cloning efficiencies the vector to insert ratio should be in the range 1:5 to 1:10. To calculate the amount of insert required when using the standard 50 ng of vector, the size of the insert (bp) was multiplied by 1.3 x 10^{-1}. This calculation is derived from the formula [(size insert (bp) x amount vector (ng)) / size vector (bp)] x (insert/vector) = ng, and gives the correct amount of insert to be used in the ligation reaction. Thus, for each PCR product to be cloned, a ligation reaction was set up by preparing a ligation mixture (Appendix D), stirring gently with a pipette tip, and incubating at 16 ^{0}C for at least 2 hr (or overnight).

The transformation procedure was then performed as follows. The required number of pMOS*Blue* competent cells were thawed and 20 µl added to pre-chilled microfuge tubes (one tube for each transformation). One µl of ligation mixture was added to the cells, stirred gently and the tubes left on ice for 30 min. The cells were then heat shocked for exactly 40 sec in a 42 ^{0}C water bath, and placed on ice for 2 min. Following this, 80 µl of room temperature SOC medium (from the kit) were added to each tube and the tubes were put on to shake (at 200-250 rpm) at 37 ^{0}C for 1 hour.

In the meantime 82 mm L agar plates, containing 50 mg/ml ampicillin (Sigma) and 15 mg/ml tetracycline (Sigma), were each spread with 35 ml of 50 mg/ml X-gal (GibcoBRL) and 20 ml 100 mM IPTG (Sigma). The plates were left to

soak for at least 30 min prior to plating with 50 µl of each transformation, and then inverted and incubated overnight at 37 ^{0}C.

The pMOS*Blue* vector allows for blue-white screening, with recombinant colonies appearing white when plated on X-gal and IPTG indicator plates. Selected white colonies were therefore tested for the presence of recombinant DNA as follows. Single white colonies were isolated and grown in Tryptone-Yeast medium. Plasmid DNA was isolated using a Promega Wizard™ Miniprep Kit. Restriction digests were carried out, using EcoR1 and Xba1 restriction enzymes. Digests were run on a standard 1% agarose gel containing ethidium bromide alongside a 1 Kb molecular weight marker (GibcoBRL) to determine the presence and size of inserts. Plasmid DNA from a recombinant colony containing a full length insert was then selected for sequencing.

2.2.2.5 Sequencing

Sequencing was carried out using the Sequenase Version 2.0 DNA Sequencing Kit (Amersham LIFE SCIENCE). First, however, the plasmid DNA was prepared as detailed below.

Plasmid DNA (containing a full length insert) was ethanol precipitated, then incubated at -20 ^{o}C for 30 min. The precipitated DNA was centrifuged for 8 min at 13 000 g, and the ethanol supernatant discarded. The resulting DNA pellet was dried, resuspended in 20 µl DEPC/H_2O. and the DNA concentration estimated by spectrophotometry. The double-stranded DNA template was then denatured, using the following alkaline-denaturation method: 0.1 volumes of 2 M NaOH and 0.1 volumes of 2 mM EDTA were added to the DNA sample, then the mixture was incubated for 30 min at 37 ^{0}C. The mixture was neutralised by adding 0.1 volumes of 3 M sodium acetate (pH 4.5-5.5). The DNA was precipitated with 2-4 volumes of 100% ethanol (-70 ^{0}C, 15 min), spun at 13000 g for 5 min and the resulting pellet washed with 2-4 volumes of 70% ethanol (-20 ^{0}C , 10 min). The DNA was then spun at 13000 g for 5 min,

the supernatant discarded, and the DNA pellet dried for 10 min at 60 ^{0}C in a vacuum oven. The (denatured) DNA pellet was resuspended in 7 µl of DEPC/H_2O, ready for use in the annealing reaction. A sequencing ladder (Appendix D) was also prepared in tandem with the samples to be sequenced.

Following purification of the DNA, the annealing reaction was performed by heating the annealing mixture (Appendix D) for 2 min at 65 ^{0}C , then slowly cooling to <35 ^{0}C over 15-30 min. The solution was given a pulse spin, and chilled on ice. Whilst the annealing mix was cooling, 2.5 µl of ddGTP, ddATP, ddTTP, and ddCTP termination mixtures (from the kit) were added to the appropriate number of microfuge tubes (4 for each sample) and pre-warmed at 37 ^{0}C.

The labelling reaction was then performed by preparing the labelling mixture (Appendix D), mixing it with a pipette tip and incubating at room temperature for 2-5 min. Termination tubes were prepared by adding 3.5 µl of labelling reaction per microfuge tubes, and the incubation of the termination reactions continued at 37 ^{0}C for 5 min. The reactions were terminated by adding 4 µl of stop solution (from the kit) to each tube. Prior to loading 4 µl per well on a sequencing gel and running until the dye front nearly reached the end of the gel (see Appendix D), the samples were heated to 75 ^{0}C for 2 min, then immediately plunged onto ice, in order to get and maintain single stranded DNA. The sequence of the PCR product was read from an autoradiograph of the gel and compared to known DNA sequences using BCN Search Launcher Nucleic Acid Sequence Searches and BLASTN.

2.2.3 *In Situ* Hybridisation (ISH)

2.2.3.1 Probe

The *in situ* hybridisation procedure was carried out with the 126-bp probe ArDNA-a described in Section 2.2.2.1. This had been relabelled at the 3' end

with digoxigenin-11-dUTP/dATP using the Boehringer Mannheim DIG Oligonucleotide Tailing Kit as detailed in Appendix D.

2.2.3.2 In situ *hybridisation method*

A standard ISH protocol for paraffin wax-embedded tissue, and relevant adjustments for cytospin preparations, are detailed in Appendix D. Optimisation of the method investigated the effect of varying the length of the proteinase K digestion stage. Following hybridisation, the presence of probe-target complexes was detected by the ISH visualisation method (Appendix D). This procedure is again based on recognition of the digoxigenin-labelled probe by specific antibody, but the method used is multilayered and thus more sensitive than that described for Southern blotting. The experiments investigating the ability of ISH with this 126-bp probe to label amoebae in cytospin preparations and within corneal tissue are detailed in Section 3.2.2.2.

2.3 Pathogenicity

2.3.1 Pathogenicity for Corneal Tissue in Organ Culture

2.3.1.1 Invasion of corneal tissue

Both rabbit and human eyebank corneas were co-incubated, epithelium side uppermost, in individual wells of 24 well plates (Costar Ltd) with *Acanthamoeba* trophozoites (axenic cultures of >95% trophozoites at 10^6/ml in isotonic organ culture medium) at 37 ^{0}C in 5% CO_2 for periods of up to 10 days. If the whole cornea plus surrounding scleral rim was used, then size considerations meant the preparation formed a cup shape in the well, especially in the case of rabbit corneas which are larger than the human cornea. If a corneal button was trephined as for grafting, then this could be fitted into an individual well without distortion, but tended to form a slight dome shape consistent with the natural curvature of the cornea. In the former case, the suspension of amoebal trophozoites tended to settle out in the hollow of the corneal cup while in the latter they tended to settle out peripheral to the corneal

button. In order to enhance the likelihood of invasion of amoebae, eyebank corneas were also incised limbus to limbus with 3-4 scalpel cuts, several mm deep, designed to breach Bowman's layer or, alternatively, simply cut into strips.

Later a more refined method of incubation was developed (Fig 2.3B); it involved incubation of an inverted human eyebank cornea, either whole or incised as described, on top of a contact lens (ETAFILLON A, VISTAKON). The contact lens, which was of the individually packaged, daily wear, disposable variety, was supported in its own holder. One ml of amoebal suspension (>95% trophozoites at 10^6/ml) was dropped onto the cupped surface of the lens prior to gently placing the cornea, epithelium facing downwards, on top. Co-incubation was conducted as before for 24 hr or 10 days. The presence of amoebae within the tissue was detected by immunolabelling with SK anti-*Acanthamoeba* antiserum using the ABC-Peroxidase technique (Appendix C). Details of the experiments performed are listed in Section 4.2.1.1.

2.3.1.2 Incubation of inoculated tissue

Prior to successful demonstration of invasion, behaviour of *Acanthamoeba castellanii* within corneal tissue, and the resulting pathology, was studied post-inoculation of amoebae into the stroma. The technique was exactly as described in Section 2.1.2.2 except that an inoculum of *either* trophozoites (>95%) *or* cysts (>95%) was utilised for migration and excystation studies respectively. The hypotonic-hypertonic method adopted allowed use of minimal force to instil the amoebae, thus minimising disruption of tissue architecture. To further maximise morphological preservation, preliminary studies had compared corneal fixation by neutral buffered formalin (a cross-linking fixative), Bouin's fluid (a coagulative fixative - Appendix E) and cetrimide (10^{-6} M, Sigma), a cationic surfactant suggested to provide rapid fixation of corneal tissue (Ball *et al.*, 1991). As no distinct superiority could be demonstrate for any of the 3 fixatives, neutral buffered formalin was used in

subsequent studies as the effect of the other two fixatives on immunodiagnostic methods or ISH was not known.

Post-inoculation of either cysts or trophozoites, each half-cornea was incubated in isotonic organ culture medium at 37 ^{0}C in 5% CO_2. After various incubation times, a strip, perpendicular to the inoculation tract, was removed for fixation. In all cases the first strip was dissected 1-2 hr post-inoculation to serve as a baseline.

Following paraffin embedding, sections were cut at 5 μm from the whole preparation. The experiments performed with this method are detailed in Section 4.2.1.2. Alternatively, in order to develop a method to preserve precious pathological specimens, core biopsies were taken using a modified renal biopsy needle (Cassella *et al.,* 1989), re-embedded and then sectioned (Fig 2.4).

2.3.2 Cytopathic Effect of Free-living Amoebae on Corneal Cells in Tissue Culture

2.3.2.1 Incubations

Experiments involving exposure of cells to amoebae were conducted on 13 mm coverslips in 24 well plates at 37 ^{0}C in a humidified incubator with 5 % CO_2. Individual coverslips could then be removed as required; preliminary experiments had shown that fixation of cells grown directly in the wells had caused cell damage in adjacent wells. Co-incubations were continued for a maximum of 10 days, due to concerns about deteriorating media quality with time.

Using 1ml of EGM 2O% per well, keratocytes were added at 50 000 keratocytes per ml per well and allowed to plate down overnight. This had been

previously established as approximately equivalent to the number of confluent epithelial cells per well in a 24 well plate. All experiments to compare the relative cytopathogenicity of different amoebae involved keratocytes as did experiments designed to elucidate the mechanisms involved; the relative susceptibility of epithelial cells and keratocytes to the cytopathic effects of *Acanthamoeba castellanii* was also investigated, both quantitatively and qualitatively.

All amoebae added to the cells were axenically (*Acanthamoeba castellanii*) or monoxenically (*Acanthamoeba castellanii*, V-EYE & H-EYE) grown trophozoites in the log phase of growth (Section 2.1.1.5) which were harvested as previously described (Section 2.1.1.6). Following resuspension at the appropriate concentration in EGM supplemented with only 2% foetal calf serum (EGM 2%) they were then added to the 24 well plates at 1 ml/well after removal of the EGM 20%. EGM 20% was thus replaced with EGM 2% while the cells were exposed to the amoebae; this was to minimise further corneal cell growth during the course of the experiment. The investigations performed with this method of co-incubation of cells and amoebae are detailed in Section 4.2.2.

2.3.2.2 Processing and image analysis

Following fixation with neutral buffered formalin, cells on coverslips were twice washed briefly in tap water, stained with Coomassie blue (Appendix E) for 5 min and washed a further twice with tap water before mounting, cell side down, with synthetic resin.

Image analysis was then performed using an MCID/4 Analyzer (Imaging Corp, St Catherine) in combination with a PowerHAD Sony 3CCD Colour Video camera and a Leitz Dialux 20EB microscope. The area of blue in each of 9 adjacent fields, arranged 3X3 in order to all but cover the whole area of the coverslip when examined under the X1.6 objective, was measured. This

arrangement had the advantage of largely allowing areas of cell damage due to handling with forceps to be excluded from the calculations. As each experiment had been performed in triplicate, the mean of the sum of these 9 areas was divided by the mean of the sum of the area of blue in the equivalent fields in the corresponding control. The value obtained thus represented the area of cells surviving, expressed as a fraction of the area of cells measured on the relevant control. This method was utilised in the experiments described in Sections 4.2.2.2 & 4.2.2.3.

2.3.3 Investigation of Method of Cytopathic Effect

2.3.3.1 Qualitative morphological studies

The mechanism of cytopathogenicity was investigated by a range of morphological studies of epithelial cells and keratocytes exposed to representatives of the 3 genera of amoebae as described above (Section 2.3.2.1). Time lapse video microscopy was performed on corneal cells (confluent epithelial cells or keratocytes at an equivalent density) in 25 cm^2 tissue culture flasks, co-incubated with amoebae trophozoites in the log phase of growth at a concentration of 10^6/ml in EGM 2% (5 ml per flask). The use of flasks instead of 24 well plates allowed the appropriate CO_2 concentration, and therefore an appropriate pH level, to be maintained in the filming chamber. A Panasonic AG 6720A TLVCR was used in combination with Axiovert 100 inverted phase microscope (Zeiss, Germany) at 80X real speed.

Coverslips of corneal cells exposed to amoebae trophozoites as described above (Section 2.3.2.1), were also prepared for light microscopy and scanning electron microscopy (SEM.) Those intended for the former were fixed in neutral buffered formalin and stained with Coomassie blue as described for image analysis (Section 2.3.2.2). Coverslips of cells destined for SEM were fixed in 0.5% glutaraldehyde (Appendix E) then washed in 4 x 5 min changes of cacodylate buffer (Appendix E), immersed in 1% osmium tetroxide ((Johnson Matthey Chemicals) in cacodylate buffer for 15 min, and washed again in 4

changes of cacodylate buffer (5 min each). They were then dehydrated through graded alcohols of 25% (5 min), 50% (5 min), 75% (5 min) and 100% (4 separate washes of 5 min each) and mounted on a stub with colloidal silver for critical point drying in liquid CO_2 (EMSCOPE CBD 750) and sputter coating with colloidal graphite (Polaron SC515 SEM Coating System, Fisons Instruments). They were examined on a Jeol 6400 scanning electron microscope.

2.3.3.2 Production of cytopathic products

Preliminary experiments involved exposure of fresh keratocytes to media conditioned by amoebae that had produced a cytopathic effect on a previous population of keratocytes. Conditioned medium was harvested from 25 cm^2 tissue culture flasks of keratocytes exposed to *Acanthamoeba castellanii* trophozoites (10^6/ml, 5 ml/flask), either overnight or for periods of up to a week. This was either frozen at -20 ^{0}C for later use, or immediately added to a fresh flask of keratocytes. Co-incubation of fresh keratocytes and conditioned medium at 37 ^{0}C in 5% CO_2 was continued for up to 10 days.

Various mechanisms of cell disruption were tried with a view to producing conditioned medium through release of intracellular contents. Naturally the presence of a water expulsion vacuole meant that hypotonic fluids, or indeed water itself, would not produce lysis. Attempts to disrupt the trophozoites using sonication (Sonicleaner 6442 AE, Ultrasonics Ltd) for periods of up to 2 hr did not produce significant lysis, as estimated by numbers of whole trophozoites present before and after sonication. Success was finally achieved by means of manual disruption in a homogenisor (Uniform, Jencons), although the process required 2-3 hours depending on the concentration of amoebae present. The required concentration of trophozoites in EGM 2% was prepared for grinding. Progress was monitored until only a few whole trophozoites were visible; these were then removed along with cellular debris by filtration through a 0.22 μM filter (MILLEX-GS®, Millipore). The resulting medium was added to keratocytes at 50 000/ml/well and incubated at 37 ^{0}C in 5% CO_2 for up to 10

days.

The ability to produce damage to keratocytes in the absence of physical contact was also investigated by the use of transwells (0.1 μM pore, Costar Ltd) in which the amoebae were suspended in a chamber, physically separated by a membrane from the cells in the well below (Fig 2.5). Preliminary experiments had indicated that 3 μM pores allowed penetration of the amoebae. They had also involved the presence of heat-killed *Klebsiella pneumoniae* in the upper chamber with the amoebae; this was discontinued when no difference could be demonstrated in the amount of cytopathic effect produced by amoebae incubated thus, compared to that produced by amoebae incubated in their absence). One ml per well of EGM 2% was again used for the course of the experiment but 0.75 ml of this was added to the cells while the amoebae at a concentration of 4 x 10^6/ml were added to the upper chamber in 0.25 ml. The final concentration of amoebae, therefore, was equivalent to 10^6/ml/well.

The presence of apoptotic cells amongst keratocytes that had been damaged by amoebae in the absence of physical contact was tested for by means of Oncor Apoptaq™ *in situ* hybridisation apoptosis detection kit. Coverslips from transwell incubations were treated according to the protocol in Appendix E.

The experiments performed to investigate the mechanism of cytopathogenicity both in the presence and absence of physical contact between the cells and amoebae, utilising the techniques described above, are detailed in Section 4.2.3.

TABLE 3.2

Indirect Immunofluorescence:
Optimal Endpoint Titres of Polyclonal Anti-amoebal Antisera

Isolate	DW Anti-*Acanthamoeba* Neff	DW Anti-*Acanthamoeba* HN3	SK Anti-*Acanthamoeba*	GRI Anti-*Acanthamoeba*	GRI Anti-*Vahlkampfia*	GRI Anti-*Hartmannella*
Acanthamoeba[1]	1:30	1:30	1:40	1:15	1:15	1:15
Acanthamoeba[2]	1:30	1:30	1:40	1:15	1:15	1:15
V-EYE	1:30	1:30	1:40	1:15	1:15	1:15
H-EYE	1:30	1:30	1:40	1:15	1:15	1.15

Code: 1-*Acanthamoeba sp* isolated from cornea of a patient with keratitis
2-*Acanthamoeba sp* isolated from contact lens case of a patient with keratitis

TABLE 3.1

Controls for ABC-Peroxidase Immunolabelling Technique

1° layer	2° layer	3° layer	Comments	Expected Result
+	+	+	+ve tissue control	Labelling
+	+	+	-ve tissue control	No labelling
+	+	+	1° preabsorbed with specific Ag	Decreased/absent labelling
+	+	+	1° preabsorbed with unrelated Ag	Labelling
Rabbit Serum	+	+	-	No labelling
Unrelated 1°	+	+	-	Labelling of unrelated Ag
+	+	-	-	No labelling
+	-	-	-	No labelling
-	+	+	-	No labelling
-	+	-	-	No labelling
-	-	+	-	No labelling
-	-	-	-	No labelling

Code: + indicates that this stage was carried out as normal
- indicates that this stage was replaced with incubation with normal serum alone

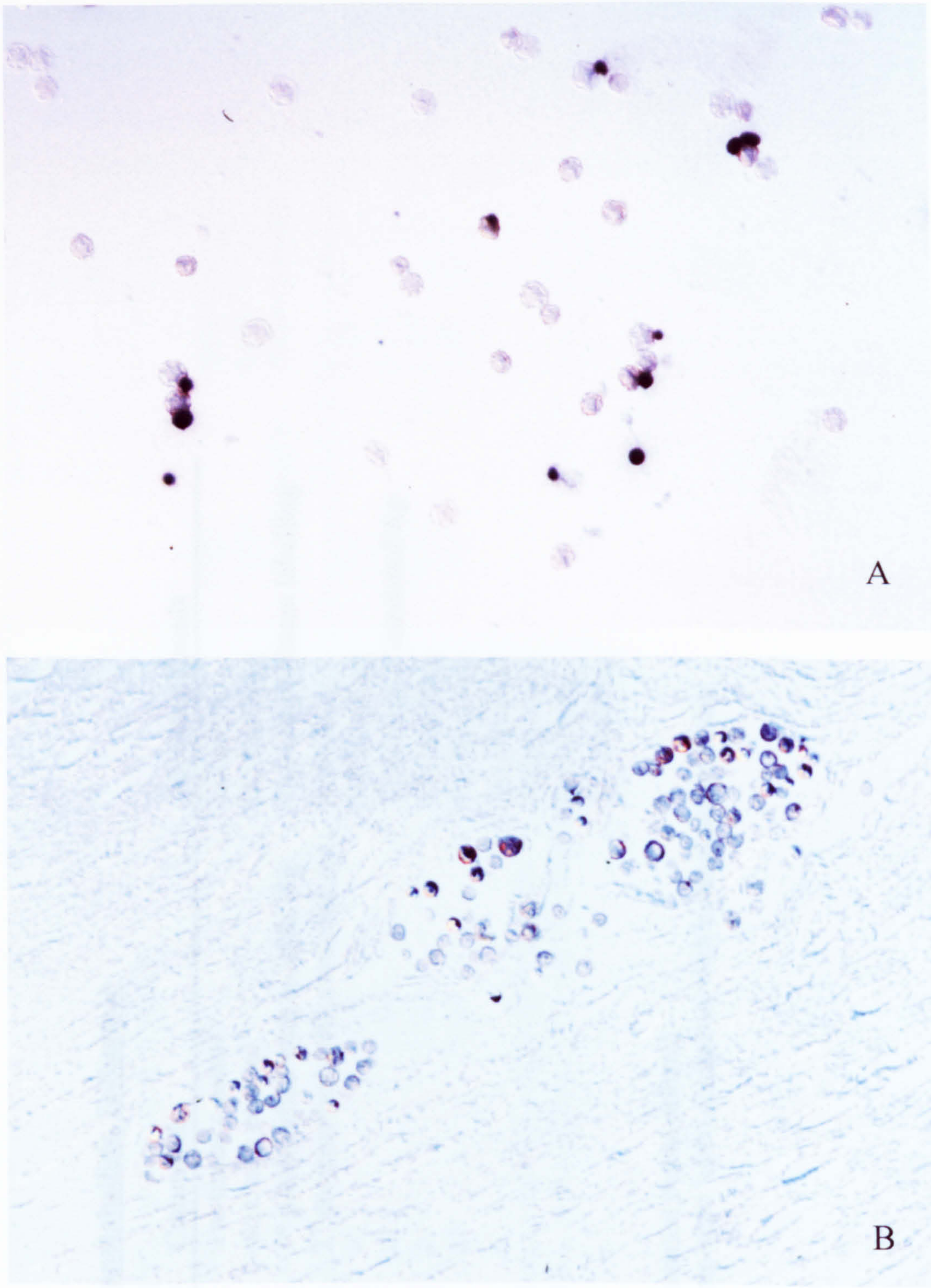


Fig 3.5 *In situ* hybridisation using 126-bp anti-*Acanthamoeba* probe

A) Cytospin preparation of *Acanthamoeba castellanii* demonstrating failure to label the cysts themselves. The significance of the heavy deposits of label just adjacent to some of the cysts is uncertain but they may represent emerging trophozoites.

B) Paraffin-embedded section of *Acanthamoeba polyphaga* demonstrating patchy labelling of the organisms. Some organisms are completely unlabelled while in other cases label is deposited only around the periphery of the cell. It is unknown whether the few organism which are heavily labelled are cysts or trophozoites.

(Interference contrast microscopy: mag x200)

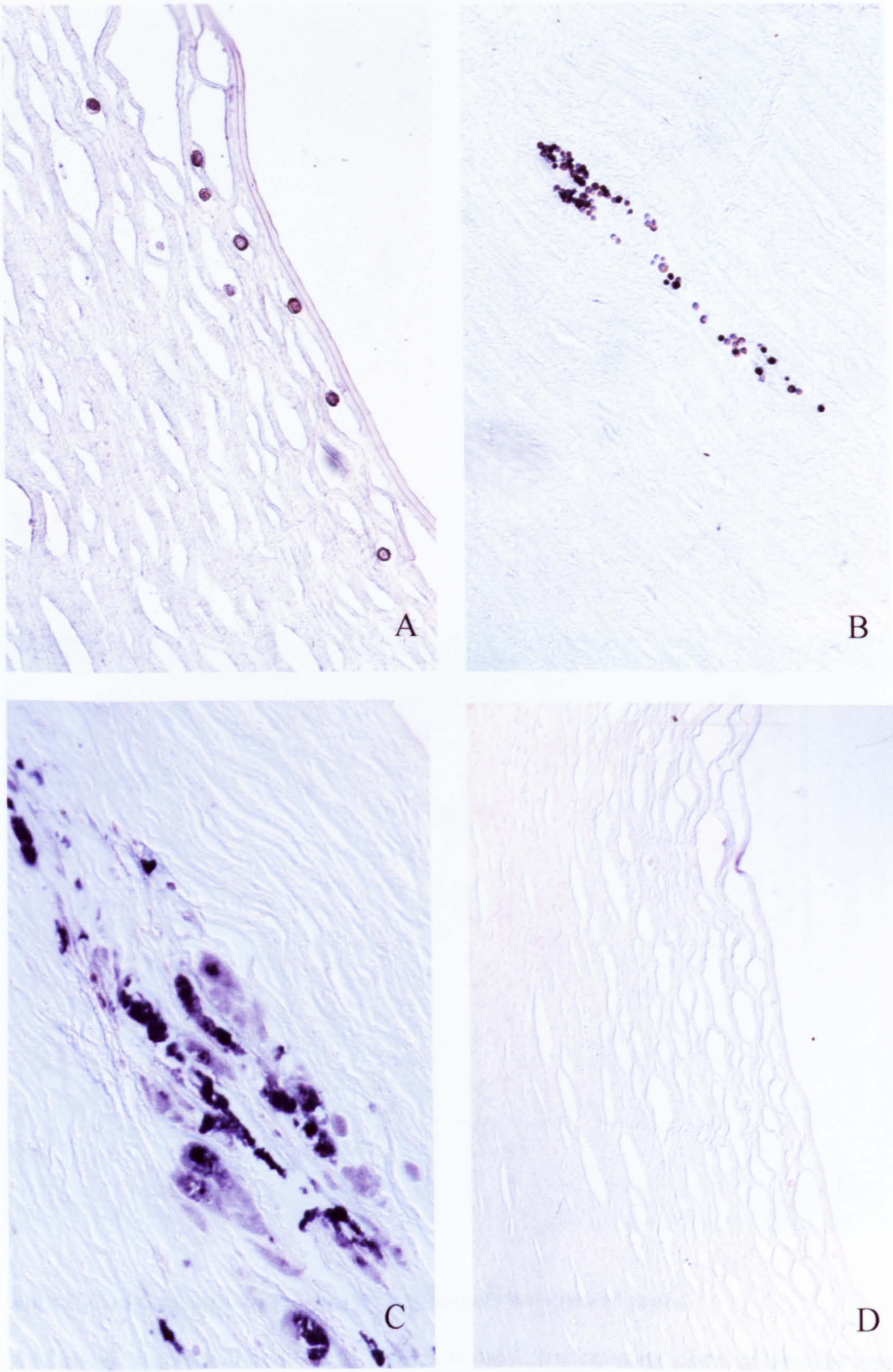


Fig 3.4 *In situ* hybridisation using 126-bp anti-*Acanthamoeba* probe

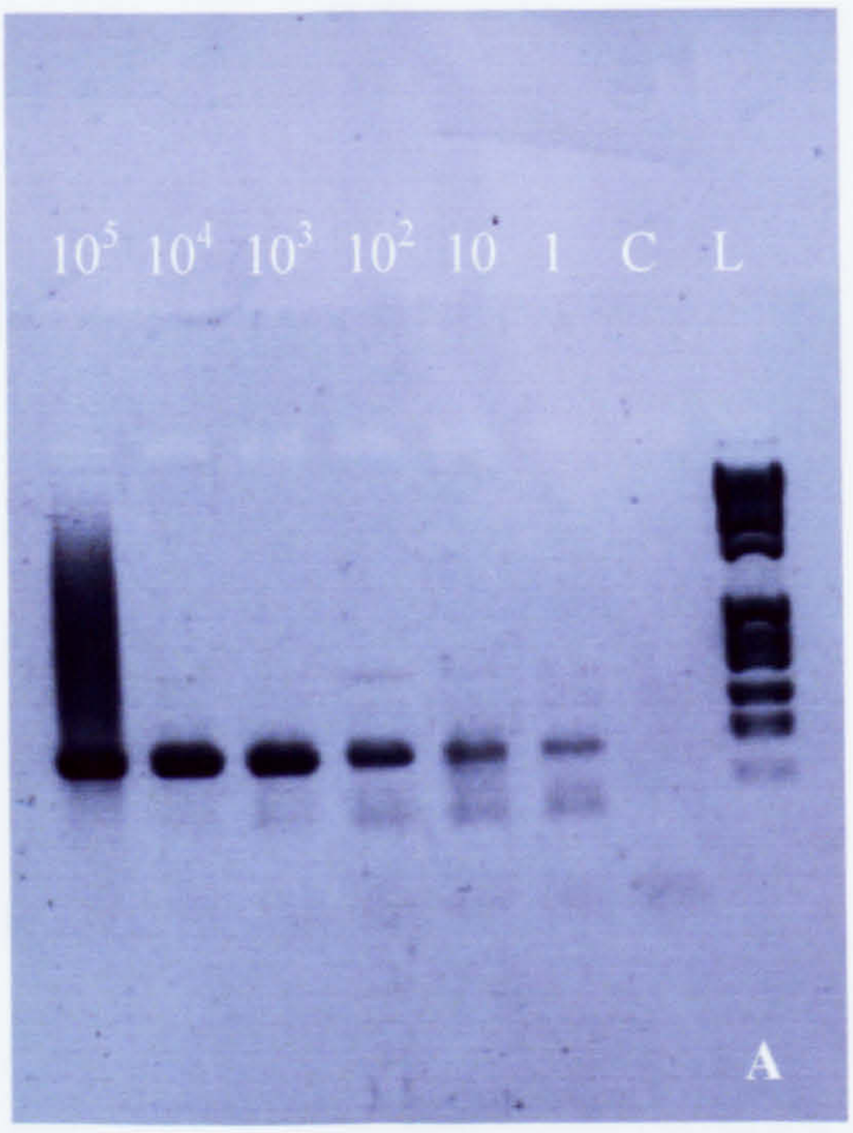
A) Labelling of *Acanthamoeba castellanii.*

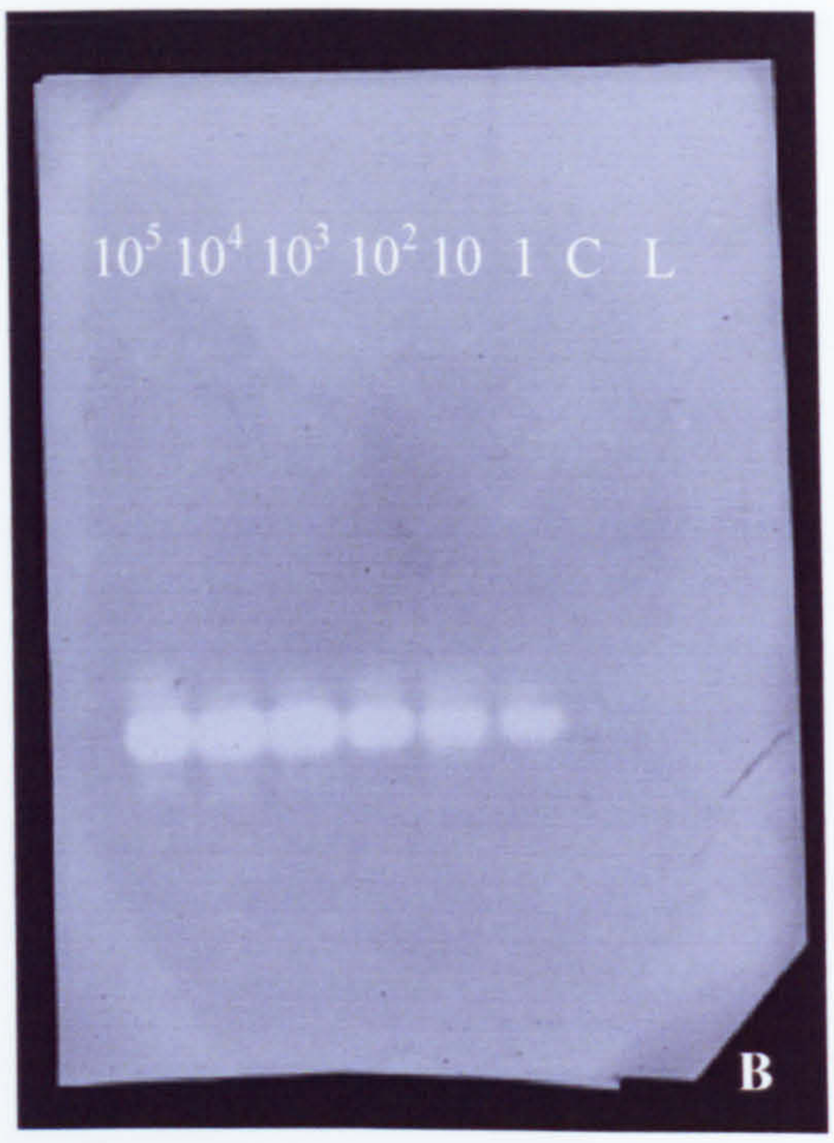
B) Labelling of H-EYE. The results for *Hartmannella vermiformis* were similarly positive.

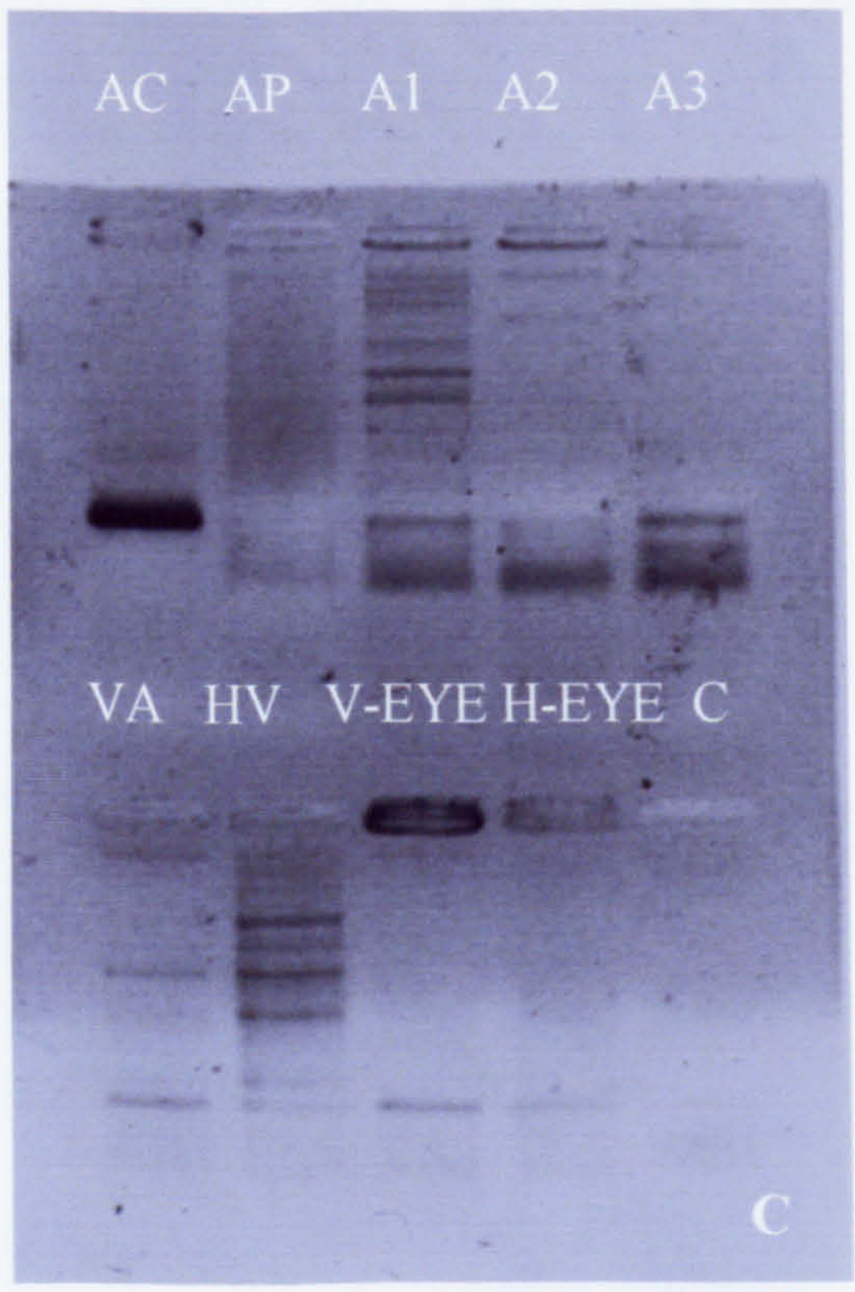
C) Labelling of *Vahlkampfia avara*. See sections 3.3.2.2 & 3.4.2.2 concerning failure to label V-EYE.

D) Negative control demonstrating no nonspecific labelling of *Acanthamoeba castellanii*. For each of the other 5 organisms, the respective negative control was similarly unlabelled.

(Interference contrast microscopy: mag x140)







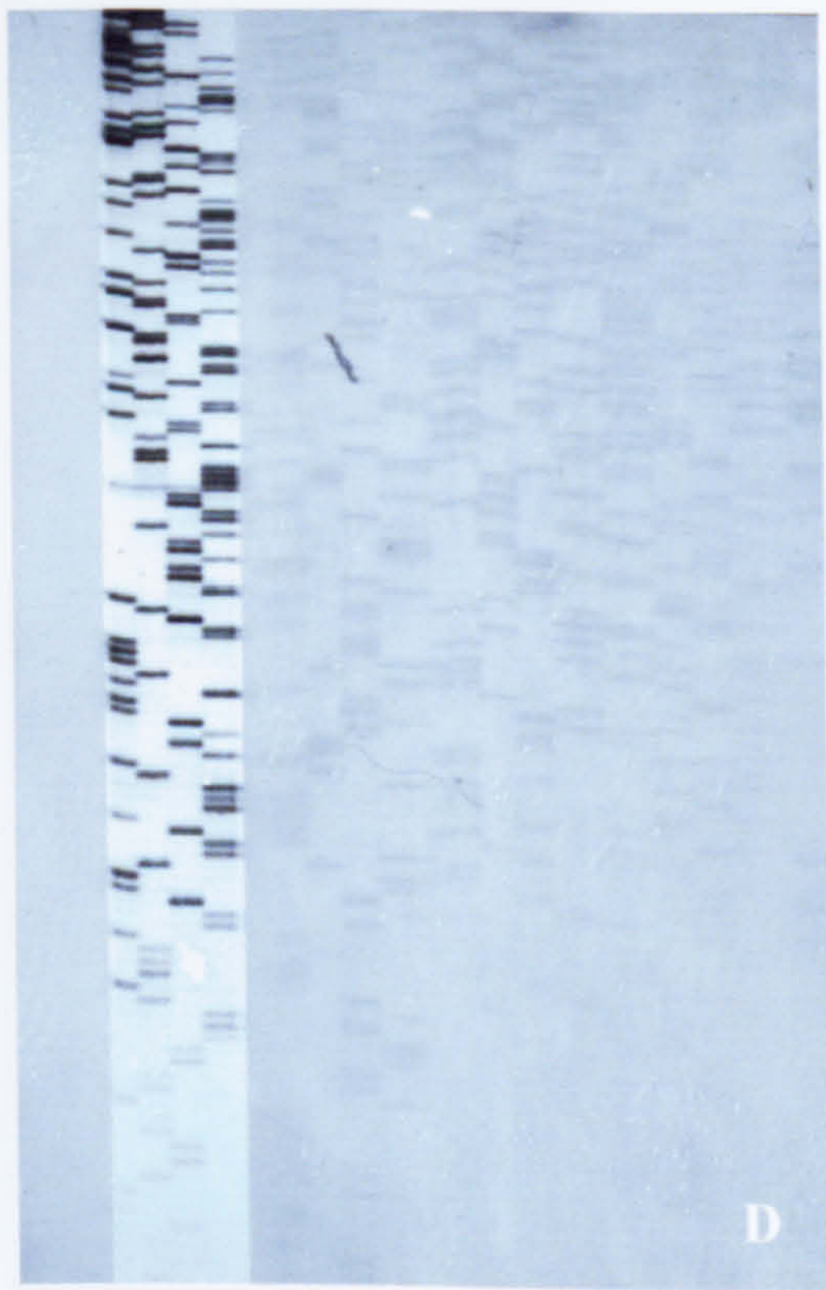


Fig 3.3 PCR with primers P1 and P2

A) PCR, performed as per standard protocol, against a range of dilutions of *Acanthamoeba castellannii* digest. The figures indicate the approximate equivalent dilution of organisms per reaction.
B) Southern blot of the PCR gel illustrated in Fig 3.3A, using 126 bp probe complementary to the expected PCR product.
C) PCR, performed as per standard protocol, against the organisms indicated, demonstrating non-specific bands.
D) Sequencing gel of the cloned product of PCR of *Acanthamoeba castellanii* digest.

(Code: C- control; L-ladder; A1,A2,A3-*Acanthamoeba sp* isolated from keratitis cases)

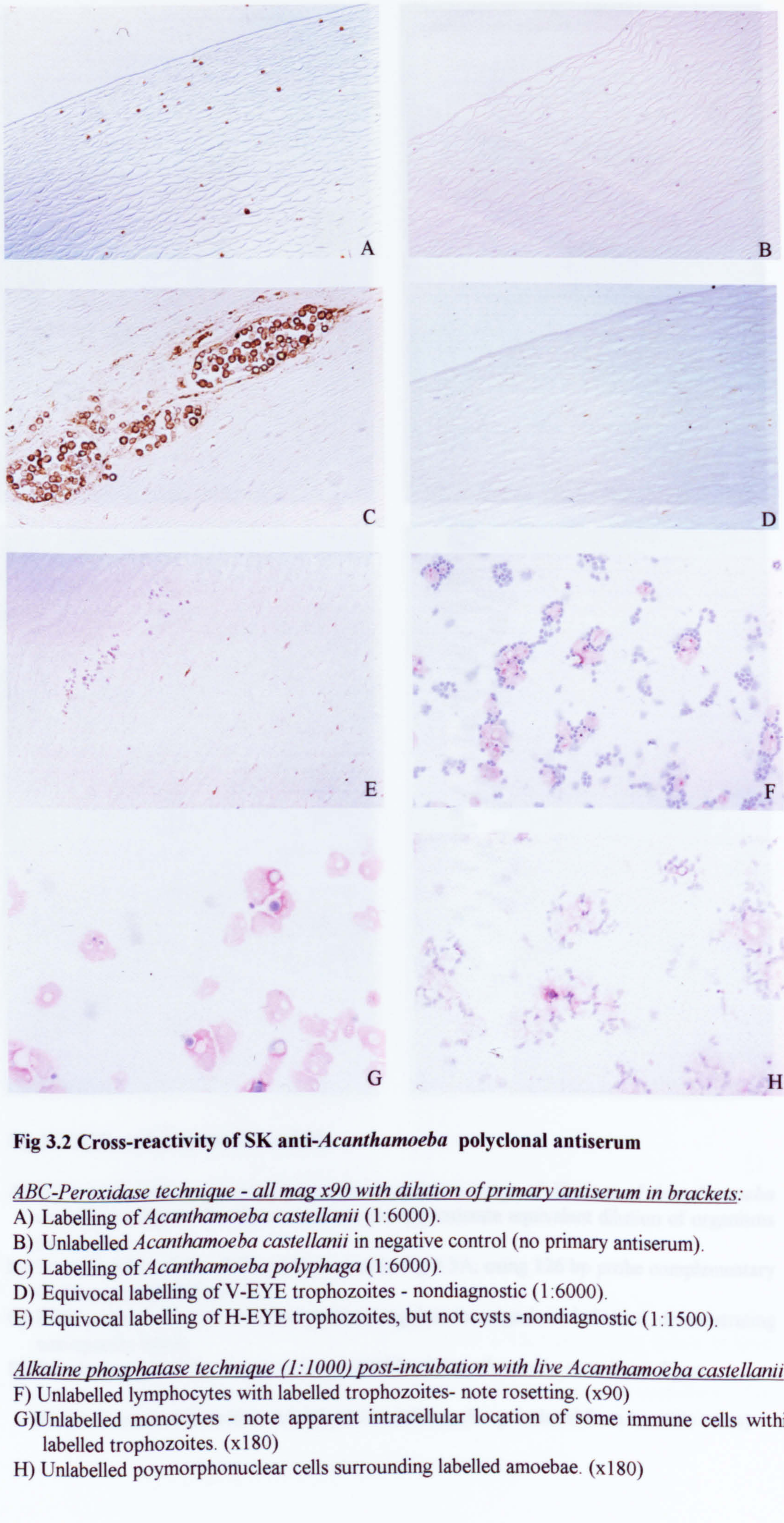


Fig 3.2 Cross-reactivity of SK anti-*Acanthamoeba* polyclonal antiserum

ABC-Peroxidase technique - all mag x90 with dilution of primary antiserum in brackets:

A) Labelling of *Acanthamoeba castellanii* (1:6000).
B) Unlabelled *Acanthamoeba castellanii* in negative control (no primary antiserum).
C) Labelling of *Acanthamoeba polyphaga* (1:6000).
D) Equivocal labelling of V-EYE trophozoites - nondiagnostic (1:6000).
E) Equivocal labelling of H-EYE trophozoites, but not cysts -nondiagnostic (1:1500).

Alkaline phosphatase technique (1:1000) post-incubation with live Acanthamoeba castellanii:

F) Unlabelled lymphocytes with labelled trophozoites- note rosetting. (x90)
G)Unlabelled monocytes - note apparent intracellular location of some immune cells within labelled trophozoites. (x180)
H) Unlabelled poymorphonuclear cells surrounding labelled amoebae. (x180)

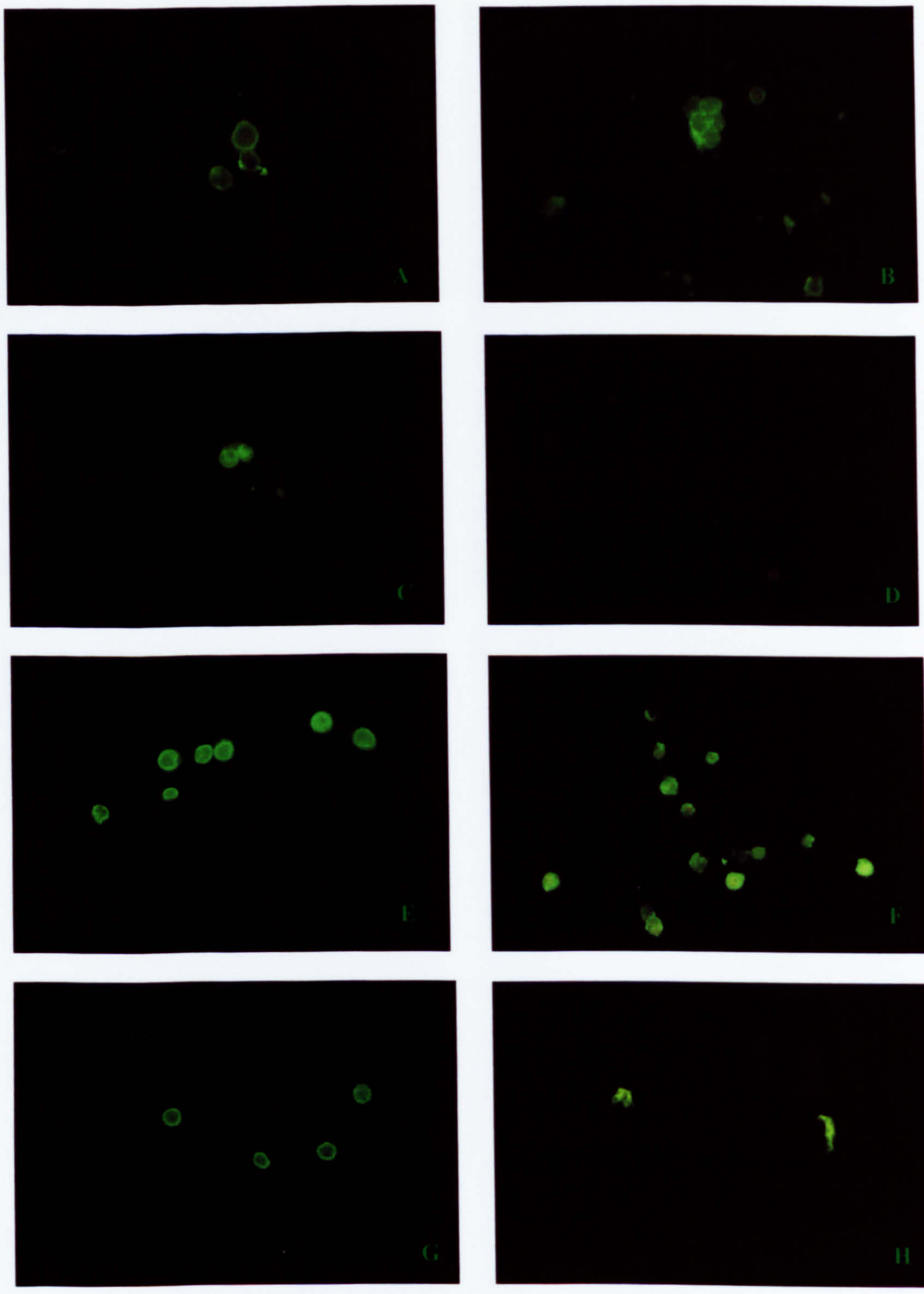


Fig 3.1 Cross-reactivity of anti-*Acanthamoeba* antisera by indirect immunofluorescence

A) *Acanthamoeba polyphaga* labelled with DW anti-*Acanthamoeba* Neff antiserum (1:30).
B) *Vahlkampfia avara* labelled with DW anti-*Acanthamoeba* Neff antiserum (1:30).
C) *Hartmannella vermiformis* labelled with DW anti-*Acanthamoeba* Neff antiserum (1:30).
D) Negative control for *Hartmannella vermiformis* of no primary antiserum demonstrating minimal background or autofluorescence post-fixation. Controls for the other 2 genera were similar.
E) *Acanthamoeba polyphaga* labelled with SK anti-*Acanthamoeba* antiserum (1:40).
F) *Vahlkampfia avara* labelled with SK anti-*Acanthamoeba* antiserum (1:40).
G) *Hartmannella vermiformis* labelled with SK anti-*Acanthamoeba* antiserum (1:40).
H) *Hartmannella vermiformis* trophozoites labelled with SK anti-*Acanthamoeba* antiserum (1:40). All the other illustrations are probably cysts, although it is difficult to be certain.

(A-H: mag x100)

3. DIAGNOSTIC METHODS

3.1 Introduction

3.1.1 Histology and Immunodiagnosis

3.1.1.1 Conventional histology

This chapter describes the ability of 3 less commonly utilised stains, namely Alcian blue critical electrolyte method, Grocott-Gomori's methenamine silver and calcofluor white, to enhance the visualisation of *Acanthamoeba* in corneal tissue. Their potential for diagnostic use is compared with 3 more standard stains, namely H&E, Giemsa and PAS.

3.1.1.2 Immunodiagnosis studies

The ability of six different rabbit polyclonal antisera (Table 2.2), to recognise *Acanthamoeba*, *Vahlkampfia* and *Hartmannella* sp in cytospin preparations is investigated by indirect immunofluorescence; 3 of these antisera were putatively specific for *Acanthamoeba* and indeed were in routine diagnostic use. On the basis of these results the highest titre antiserum is then selected for more in-depth study. Determination of the optimal dilution of this antiserum using the ABC-Peroxidase technique is detailed for representatives of the 3 genera of amoebae. Utilising this antiserum and the indirect alkaline phosphatase technique, investigation of potential cross-reactivity with corneal tissue, and with inflammatory cells which had been pre-exposed to *Acanthamoeba castellanii,* is also described.

3.1.2 Molecular Biology Techniques

3.1.2.1 PCR studies

This chapter investigates the potential of primers P1 and P2, detailed in the literature (Lai *et al.,* 1994), for diagnostic PCR in cases of suspected amoebal keratitis. Confirmation of the ability of these primers to amplify DNA target

sequences from *Acanthamoeba castellanii,* both by Southern blotting with the complementary 126-bp probe ArDNA-a and by cloning and sequencing of the PCR product, is described. Assessment of the sensitivity of the method following optimisation of the PCR protocol is outlined, as is the potential for diagnostic use in the presence of corneal tissue. Cross-reactivity with representatives of the 3 genera of amoebae is then investigated by both 'hot' and 'cold' PCR.

3.1.2.2 ISH studies

Investigation of the ability of the labelled 126-bp probe complementary to the PCR product to demonstrate *Acanthamoeba castellanii* in corneal tissue, with optimisation of a standard ISH protocol for this application, is described. With a view to potential diagnostic use on epithelial scrape preparations, assessment of the applicability of this method to cytospin preparations is also outlined. The cross-reactivity of this probe with other *Acanthamoeba* sp and with representatives of the *Vahlkampfia* and *Hartmannella* genera is then investigated on inoculated corneal preparations.

3.2 Materials and Methods

3.2.1 Histology and Immunodiagnosis

3.2.1.1 Conventional histology

The ability of Alcian blue critical electrolyte concentration, Grocott-Gomori's methenamine silver and calcofluor white techniques (Section 2.2.1.1) to demonstrate *Acanthamoeba* in corneal specimens was compared with histology techniques (H&E, Giemsa and PAS) routinely used in the Western Infirmary Pathology Department for the diagnosis of *Acanthamoeba* keratitis. All 6 techniques were applied to routine histology specimens containing trophozoite and cyst forms of the organism fixed in neutral buffered formalin. Calcofluor white was also applied to *Acanthamoeba* sp in glutaraldehyde-fixed pathological corneal specimens and formalin-fixed inoculated corneas and also

unfixed cytospin preparations. The effects of pretreatment with trypsin (0.1% in PBS for 10 min at 37 ^{0}C or 0.25% for 30 min) were assessed for both glutaraldehyde- and formalin-fixed tissue.

3.2.1.2 Immunodiagnosis studies

In order to assess specificity, the ability of each of the 6 different anti-*Acanthamoeba* antisera (Table 2.2) to label cytospin preparations (Section 2.1.2.1) of representatives of the 3 genera of amoebae (Table 3.2) was tested by indirect immunofluorescence (Section 2.2.1.2). The slides were assessed by an independent panel of 'blind' observers. The controls were i) no primary antibody, ii) no secondary antibody and iii) neither primary nor secondary antibody.

SK anti-*Acanthamoeba* has currently been adopted for routine diagnostic use in the Pathology Department, Glasgow University: it is used as the primary layer in combination with an indirect alkaline phosphatase method (Section 2.2.1.2) for smear preparations or, alternatively, in combination with the ABC-Peroxidase technique (Section 2.2.1.2) if applied to paraffin-embedded corneal samples. Prior to diagnostic use, the optimal dilution of the SK anti-*Acanthamoeba* antibody for *Acanthamoeba castellanii* was therefore assessed for the indirect alkaline phosphatase method on cytospin preparations (Section 2.1.2.1). The controls included were i) no primary antibody, ii) no secondary antibody and iii) neither primary nor secondary antibody. Furthermore, the optimal dilution for the 6 representatives of the 3 different genera of amoebae (Table 2.1) was assessed for SK anti-*Acanthamoeba* using the ABC-Peroxidase technique on paraffin-wax embedded sections (Section 2.1.2.2). The controls included are detailed in Table 3.1.

The postulated cross-reactivity of SK anti-*Acanthamoeba* with inflammatory cells in the cornea, and indeed with the corneal cells themselves, was investigated using the indirect alkaline phosphatase technique (Section 2.2.1.2).

This antisera was tested against epithelial cells and keratocytes (on coverslips), and cytospin preparations of monocytes, lymphocytes and polymorphonuclear leucocytes, both *de novo* and following exposure to *Acanthamoeba castellanii* (10^6/ml, and in certain cases serial ten-fold dilutions of 10^6/ml, for 24 hr at 37 ^{0}C in 5% CO_2 in hypotonic eyebank medium). One possible explanation for the proposed cross-reactivity of anti-*Acanthamoeba* antisera is that the immune cells contained ingested amoebae fragments. The *Acanthamoebae* to which the cells were exposed were therefore variously i) live, or ii) incapacitated by addition of specific polyclonal antisera DW anti-*Acanthamoeba* HN3 (50 µl in 2.5 ml of 10^6/ml *Acanthamoeba castellanii*), prior to incubation with the cells, or iii) killed, either by autoclaving or by methanol fixation. This was to minimise the damage inflicted on the other cells by the amoebae, in order to tip the balance in favour of those immune cells capable of themselves inflicting damage and ingesting the amoebae.

3.2.2 Molecular Biology Techniques

3.2.2.1 PCR studies

DNA was extracted by proteinase K digestion (Section 2.1.2.3) from the amoebae listed in Table 2.1. to produce a final concentration equivalent to 10^5 amoebae per 5µl reaction volume. The amoebae, which were grown on heat killed bacteria on non-nutrient agar (Section 2.1.1.2), were harvested as >95% trophozoites, except in one case where >95% cysts of *Acanthamoeba castellanii* were used. DNA extracts from heat-killed bacteria were therefore also prepared, as well as extracts from corneal tissue and cultured keratocytes and corneal epithelial cells.

Initially, standard cold PCR (Section 2.2.2.2) using upstream primer P1 and downstream primer P2 from the 5' ends of both strands of the ArDNA-a locus (Section 2.2.2.1) was performed on *Acanthamoeba castellanii* DNA extract, based on the protocol described by Lai *et al.* (1994).

As a one-off procedure, in order to confirm its identity, the PCR product was cloned using the pMOS*Blue* t-vector Kit RPN 1719 (Section 2.2.2.4) and then sequenced by the dideoxynucleotide chain termination method using Sequenase Version 2.0 DNA Sequencing Kit (Section 2.2.2.5). The sequence obtained was analysed and compared with known sequences in EMBL-Genbank databases using BLAST search (Altschul *et al.*, 1990) and the National Centre for Biotechnology Information's BLAST WWW server with Entrez and SRS links (provided by Human Genome Center, Baylor College of Medicine). Thereafter, identity of the PCR product was confirmed by Southern blotting followed by hybridisation (Section 2.2.2.3) with the digoxigenin-labelled complementary 126-bp probe (Section 2.2.2.1).

The sensitivity of the adapted method was assessed using serial ten-fold dilutions of the target organism. The ability to detect cysts was also investigated with DNA extracted from >95% *Acanthamoeba castellanii* cysts. Potential cross-reactivity was tested for with DNA extracts from heat killed bacteria, corneal tissue, epithelial cells and keratocytes. The optimised PCR method was then tested against DNA extracts from the 6 representatives of the 3 genera of amoebae (Table 2.1). The effect of altering the stringency of the PCR conditions was assessed by increasing and decreasing the annealing temperatures and by decreasing the magnesium concentration. The effect of altering the gel running conditions, with regard to the detection sensitivity of the method, was investigated by varying the ethidium concentrations and the electrophoresis time. 'Hot' PCR (Section 2.2.2.2.) was also performed on these samples so that the products could be run and visualised on a polyacrylamide gel, which is capable of differentiating DNA fragments which differ by only one base pair.

3.2.2.2 ISH studies

ISH (Section 2.2.3.2) was performed using the 126-bp probe (Section 2.2.3.1) labelled with digoxigenin 11-dUTP/dATP (Section 2.2.3.1) on both cytospin preparations (Section 2.1.2.1) and paraffin wax-embedded corneal tissue

preparations (Section 2.1.2.2) of the 6 representatives of the 3 genera of amoebae (Table 2.1). Optimisation of the standard protocol involved altering the severity of the proteinase K digestion stage by utilising incubation times of 20, 25, 30, 35 and 40 min.

3.3 Results

3.3.1 Histology and Immunodiagnosis

3.3.1.1 Conventional histology

Alcian blue critical electrolyte method demonstrated the presence of both cysts and trophozoites of *Acanthamoeba* poorly. Grocott-Gomori's methenamine silver stain similarly demonstrated both forms of amoebae no better that the histology techniques in routine use.

Calcofluor white enabled reasonable visualisation of *Acanthamoeba* cysts in cytospin preparations and in corneal tissue but only poorly demonstrated the presence of trophozoites. The cysts, which are readily recognisable by their distinctive morphology in well preserved specimens, fluoresced strongly apple-green. The trophozoites, however, fluoresced only weakly reddish-brown. The corneal stroma also fluoresced dull reddish-brown. Following trypsinisation (0.1% for 10 min and 0.25% for 30 min), no real improvement in trophozoite visualisation was apparent for glutaraldehyde- or formalin-fixed specimens; indeed, if anything the results were better without pretrypsinisation, particularly for formalin fixation.

3.3.1.2 Immunodiagnosis studies

All 6 antibodies were capable of recognising the 3 different genera of amoebae as demonstrated by indirect immunofluorescence (Table 3.2 & Fig 3.1). It was noted that amoebae exhibit autofluorescence; this, however, was quenched by acetone fixation. Corneal tissue also demonstrates autofluorescence. The

indirect immunofluorescence method was, however, never successfully applied to paraffin wax-embedded sections in this study.

For optimal demonstration of *Acanthamoeba castellanii* by SK anti-*Acanthamoeba*, the indirect immunofluorescence (1:40 on cytospin preparations) and indirect alkaline phosphatase methods (1:1000 on cytospin preparations) required higher concentrations of primary antisera compared to the ABC-Peroxidase technique (1:6000 on paraffin-embedded sections). Also, although the optimal dilution of SK anti-*Acanthamoeba* by indirect immunofluorescence clearly labelled *Vahlkampfia* and *Hartmannella* sp, this was not the case with the same antiserum in combination with the ABC-Peroxidase technique. Using this technique, demonstration of *Vahlkampfia* and *Hartmannella* sp required higher concentrations of primary antibody (Table 3.3 and Fig 3.2A-E). This was particularly marked with cyst forms of the organisms (Fig 3.2E). The results listed in Table 3.3 refer to one particular antibody run: although the pattern was consistent as regards differentiated labelling of the 3 genera, there was run-to-run variation with respect to the optimal dilution for a specific genera.

Using the ABC-Peroxidase technique, non-specific labelling of corneal tissue, particularly epithelium, and also of co-inoculated heat killed bacteria, was observed. This was most marked in areas of tissue disruption and with higher concentrations of primary antibody. Investigation of potential cross-reactivity, using SK anti-*Acanthamoeba* antisera in combination with the indirect alkaline phosphatase method, did not demonstrate labelling of cultured epithelial cells and keratocytes or of lymphocytes, monocytes and polymorphonuclear cells (Fig 3.2F-H). This was the case whether the cells were *de novo* or had been exposed to live, killed or incapacitated *Acanthamoeba*.

3.3.2 Molecular Biology Techniques

3.3.2.1 PCR studies

PCR amplification of DNA extracted from various numbers of *Acanthamoeba castellanii* cells (10^5, 10^4, 10^3, 10^2, 10^1 and 1), with primers P1 and P2 resulted in a single PCR band at the expected 122-bp position in each case (Fig 3.3A). There was some run-to-run variation in the sensitivity of the method. DNA from control cells (*Klebsiella aerogenes*, cornea, cultured keratocytes and cultured corneal epithelial cells) demonstrated no detectable amplification of the target sequence using the same conditions.

Southern blotting and hybridisation with the complementary 126-bp probe described gave a positive result (Fig 3.3B). Sequencing the PCR product (Fig 3.3D) confirmed that it was derived from the *Acanthamoeba castellanii* ArDNA-a region of the rDNA locus.

All of the other species of amoebae tested under the same conditions and at the same concentration (10^4) failed to give an unequivocally positive band at the 122-bp position (Fig 3.3C). In some cases a faint ghost band was produced, often in conjunction with other smaller non-specific bands. All these bands gave a positive result following Southern blotting and hybridisation. There was run-to-run variation in both the production of a ghost band at the 122-bp position, and the other non-specific bands, for any given specimen. Furthermore, the sensitivity of the detection method could be decreased by decreasing the ethidium bromide concentration, or by running the electrophoresis for longer (which effectively decreased the ethidium bromide concentration).

Decreasing the annealing temperature (58^0C, 55^0C and 50^0C), and so making the conditions less stringent, resulted in more non-specific bands. Increasing the annealing temperature (63^0C and 65^0C), and so making the conditions more

stringent, resulted in fewer non-specific bands and also the disappearance of some of the faint ghost bands at the 122-bp position. Halving the magnesium concentration, and so making the conditions more stringent, resulted in loss of 122-bp band in the case of *Acanthamoeba castellanii* with retention of some of the non-specific bands produced by amoebae other than *Acanthamoeba castellanii.* Performing 'hot' PCR so that the PCR product could be run on a polyacrylamide gel revealed further non-specific bands not visualised on the agarose gel (polyacrylamide gels are capable of distinguishing DNA products with differ only 1-bp position in length). It also demonstrated that the faint ghost bands were all at the 122-bp position.

3.3.2.2 ISH studies

Optimisation of a standard ISH method indicated that a relatively prolonged proteinase K digestion stage of 30 min or more was required. With this modification the 126-bp probe recognised 5 out of 6 of the representative organisms listed in Table 2.1 (Fig 3.4). V-EYE trophozoites did not label; no cysts were present in this particular preparation. On top of run-to-run variation there was also substantial within-run variation of labelling of individual organisms. This was particularly marked with cytospin preparations. Trophozoites were generally labelled strongly although it may be difficult to ascertain the form of the underlying organism when obscured by heavy label. Labelling of cysts was poor and consisted, not of the expected cytoplasmic labelling, but of a thin layer of label around the cyst wall in tissue sections or patchy deposits adjacent to cysts in cytospin preparations (Fig 3.5).

3.4 Discussion

3.4.1 Histology and Immunodiagnosis

3.4.1.1 Conventional histology

The Alcian blue critical electrolyte technique uses different concentrations of $MgCl_2$ to differentially identify various acid mucins. It had not previously been

used to attempt to identify amoebae in pathological specimens but the results were unremarkable. Grocott-Gomori's methenamine silver stain recognises polysaccharides converted to dialdehydes by oxidation. As described by Theodore *et al.* (1985), Grocott-Gomori's methenamine silver outlined the cyst wall but did not stain the cytoplasm of the organism. Compared to H&E, PAS and Giemsa, these two techniques demonstrated both trophozoites and cysts only poorly and are also not generally performed as routine stains.

For both cytospin and paraffin-embedded sections, calcofluor white staining produced cysts that fluoresced apple-green. This agrees with the results of Wilhelmus *et al.* (1986) on corneal smears and Silvany *et al.* (1987) on paraffin-embedded tissue sections. Both trophozoites and corneal stroma appeared dull reddish-brown. Dull green fluorescence has, however, also been described for calcofluor white treated corneal stroma (Marines *et al.*, 1987). These effects are due to the presence of Evans' blue which reduces non-specific background fluorescence. Trophozoites fail to stain with calcofluor white as they lack chitin/cellulose in their cell walls. They are thus difficult to distinguish from other cells present in the corneal stroma. In my experience digestion with trypsin did not enhance visualisation of trophozoites. This is in contrast to the results of Silvany *et al.* (1987) who describe red-orange trophozoites standing out against a dull brick-red stromal background post-trypsinisation (0.25% for 30 min). In conclusion, therefore, reliable demonstration of the trophozoite form of amoebae by non-specific stains is still awaited.

3.4.1.2 Immunodiagnosis studies

Both cyst and trophozoite forms of amoebae were visualised by specific antisera, as previously described for *Acanthamoeba* (Blackman *et al.*, 1984, Epstein *et al.*, 1986). Preliminary indirect immunofluorescence studies on corneal isolates demonstrated this for all 3 genera of amoebae with all 6 antisera, indicating cross-reactivity. Although indirect immunofluorescence is a rapid and simple technique, it has a number of drawbacks. Its relative

insensitivity is compounded by the fact that the autofluorescence of the cornea has a masking effect. Also, results must be documented photographically as the immunofluorescence is not permanent but dissipates with time. Horseradish peroxidase or alkaline phosphatase methods of antigen detection, which produce a permanent colour change in a target chromogen, are therefore preferred for diagnostic use.

SK anti-*Acanthamoeba* was chosen for more detailed study because the optimal working dilution was higher than for the other antisera as demonstrated by indirect immunofluorescence. Although using the ABC-Peroxidase technique this antiserum still cross-reacted with the 3 genera of amoebae, differences in optimal dilution were demonstrated. This afforded the theoretical possibility that at least this antiserum could be made specific by meticulous titration of dilutions and careful use of appropriate controls.

The ABC-Peroxidase technique (Hsu *et al.*, 1981) was utilised for more detailed studies with SK anti-*Acanthamoeba* because it is more sensitive than the other methods: this is presumably due to the amplifying effect of 3 different sequentially- applied layers in combination with the multi-valency of the third layer. In practice this means that, for a given antiserum the optimal working dilution is higher than for less sensitive techniques. A higher dilution should effectively enhance specificity by diluting out less concentrated non-specific antibody. Theoretically this should make any difference in optimal labelling dilution between the different genera more marked and indeed this was observed (Fig 3.2 A-E, Table 3.3). In practice, however, run-to-run variation is sufficiently marked to make use of different optimal labelling dilutions for differential diagnosis of the three genera of amoebae unfeasible in the diagnostic setting.

Using the indirect alkaline phosphatase method, no cross-reactivity of SK anti-*Acanthamoeba* antisera with cultured epithelial cells or keratocytes, nor with

lymphocytes, monocytes or polymorphonuclear cells, was demonstrated. This was the case whether or not the cells had been exposed to *Acanthamoeba castellanii* under a range of conditions. Although these treatments (Section 3.2.1.2) were designed to favour immune cells ingesting amoebae, it is not certain whether in fact this occurred; numerous examples were observed, however, where trophozoites appeared to be ingesting immune cells (Fig 3.2G). It was also noted that both autoclaving and methanol fixation resulted in loss of antigenicity. This would suggest that it would be unlikely that ingested amoeba fragments within phagolysesomes would retain antigenicity.

Despite these results, labelling of surrounding tissue, especially epithelium was, however, sometimes observed in pathological specimens. This occurred in spite of the usual precautions to decrease non-specific background label and so increase signal-to-noise ratio. Since polyclonal antibodies are heterogeneous, such antisera may contain antibodies to natural hazards previously encountered by the immunised animal. It is possible that this may account for the labelling of heat-killed bacteria inoculated into the corneal tissue along with the amoebae. Theoretically, however, such specific, but unwanted, activity should be effectively removed by using high dilutions of antibody. Damaged tissue in particular subjectively appeared to attract higher levels of non-specific labelling. It was also felt that trypsinisation, used to partially digest glutaraldehyde-fixed specimens to enhance antigenicity, appeared to increase levels of non-specific labelling. All these factors suggest that such labelling is indeed non-specific background labelling rather than true cross-reactivity.

There was also marked run-to-run variation as regards the degree of such background in a given specimen. Background appeared to increase with increasing age of the SK anti-*Acanthamoeba* antibody aliquot. Careful storage of small aliquots of antibody at -20 ^{0}C with avoidance of repeated freeze-thaw cycles (i.e. good laboratory practice) is therefore important.

Other steps taken to decrease background activity include preliminary blocking of the tissue section and dilution of secondary antibody with normal serum from the species donating the secondary antibody in order to inhibit binding of immunoglobulin to Fc receptors and reaction between secondary antibody and tissue immunoglobulins. Use of an appropriately diluted complex also minimises the problem of non-specific binding associated with hydrophobic or electrostatic forces. Streptavidin is associated with particularly low background non-specific ionic interactions as its isoelectric point is nearly neutral. Furthermore, use of 3% hydrogen peroxide prior to the ABC-Peroxidase technique is essential to decrease endogenous peroxidase. Similarly intrinsic biotin, an important vitamen for transcarbamylation, may also produce unwanted staining, making interpretation difficult in the absence of carefully planned controls. This, however, was not an issue with corneal tissue in my experience.

3.4.2 Molecular Biology Techniques

3.4.2.1 PCR studies

Ribosomal DNA is multicopy and consists of highly conserved regions and variable regions. Choice of a multicopy gene such as rDNA should enhance sensitivity. Choice of a variable region as the target sequence should enhance the chances of specificity. No rDNA sequences were found to be homologous to those of *Acanthamoeba* ArDNA-a in the sequence databases (Lai *et al.*, 1993). Although the *Acanthamoeba* 26S rDNA contains regions showing a high degree of homology with other organisms, the hypervariable region present was thought to be specific enough, at least in theory, to enable differentiation between *Acanthamoeba* and other genera (Lai *et al.*, 1994). It should be noted, however, that the 26S rDNA sequences for other species of *Acanthamoeba* are not published, nor are those for *Vahlkampfia* nor *Hartmannella.*

A PCR-based strategy to detect members of the genus *Acanthamoeba* and

distinguish pathogenic strains from nonpathogenic strains using primers designed to recognise the 18S rDNA, has also been described (Vodkin *et al.*, 1992). The 26S rDNA primers P1 and P2 were preferred for this study, however, because they were not designed to be species-specific or pathogen-specific. Firstly, the taxonomy of the *Acanthamoeba* genus is still confused. Secondly, it is unclear whether pathogenic strains are restricted to certain species or whether all species are potentially pathogenic. Thirdly, keratitis is now known to be associated not only with *Acanthamoeba,* but also with *Vahlkampfia* and *Hartmannella.*

PCR amplification with P1 and P2 under the conditions described produced only the specific 122-bp product from *Acanthamoeba castellannii* crude cell digests. The cellular detection limit was 10 cells or less. This, therefore, is an exceedingly sensitive method for detecting the presence of *Acanthamoeba castellanii.* Its applicability in practice for diagnosis is, however, doubtful.

Firstly, false positive results due to contamination were problematic (review Kwok *et al.*, 1989) in spite of the precautions taken (Section 2.2.2.2). *Acanthamoeba* are ubiquitous in the environment and, as demonstrated, exponential amplification, and choice of a multicopy target sequence, makes the method exquisitely sensitive. Furthermore, *Acanthamoeba* may be present on the tear film without being implicated in causing disease (Dr John Hay - personal communication). Obviously, in a diagnostic scenario, all of these factors would make interpretation of the significance of a positive result difficult. This is particularly problematic because the result is dependent simply on the absence or presence of a band in a gel [see reviews by Wright & Wynford-Thomas (1990) and Stoker (1990)]. ISH may therefore be a more appropriate technique for diagnosis because it is based on demonstration of the organism *in situ* in the tissue.

Secondly, the ability of PCR with the primers and conditions described to

detect amoebae other than *Acanthamoeba castellanii* is uncertain. The results of Lai *et al.* (1994) suggest that the test is indeed specific for all *Acanthamoeba,* but not other genera of amoebae. They had not, however, tested *Hartmannella* and *Vahlkampfia.* The data presented here, however, shows a strong positive result for *Acanthamoeba castellanii* but not for the other *Acanthamoeba* sp tested, nor for the *Hartmannella* sp or *Vahlkampfia* sp tested.

The significance of the faint ghost band produced at the 122-bp position is also uncertain. Run-to-run variation compounded the difficulties in interpretation. It may simply be present due to low level contamination with *Acanthamoeba castellanii* DNA, in spite of the precautions outlined. It may also be the result of slight mismatch with the primers for the appropriate sequence of rDNA in these related species and genera of amoebae, resulting in variable low-level amplification products. The polyacrylamide gel results, however, suggest that the faint ghost band itself is exactly 122-bp in length.

No set of conditions were found which made the method capable of: i) detecting all amoebae tested; or ii) detecting all *Acanthamoebae* tested but not other genera of amoebae; or iii) detecting only *Acanthamoeba castellanii.* Furthermore, although there were differences, analysis of the non-specific bands produced did not reveal any consistent pattern which might permit differentiation of the different species or genera. These factors would obviously limit the usefulness of the test in a diagnostic setting. They contrast, however, with the results of Vodkin *et al.* (1992), who describe PCR with primers against 18S rDNA designed to recognise *Acanthamoeba polyphaga,* which, under less restrictive annealing conditions, produced reproducible and distinct banding patterns for all species except *Acanthamoeba polyphaga.* They also claim that this multiple banding pattern can, in certain cases, differentiate pathogenic from non-pathogenic strains. (see, however, Section 1.1.1 & Section 1.1.2 concerning uncertainties related to taxonomy and designation of pathogenic strains).

3.4.2.2 ISH studies

This technique has not previously been utilised to demonstrate the presence of protozoa in tissue. Although 5 out of 6 of the organisms representing the 3 genera were successfully labelled, it currently offers no improvement over antibody techniques as the same cross-reactivity with all 3 genera of amoebae was observed with more patchy within-run variation. Also, for some unknown reason, V-EYE apparently failed to label; this may be related to the presence of a mucoid coat (Fig 2.1B). Furthermore, in contrast to non-specific staining techniques, the cysts were generally poorly demonstrated compared to trophozoites (Fig 3.5). In the former only a thin deposit of label was observed around the cyst wall, while in the latter the expected cytoplasmic labelling was marked. The effect was even more marked with cytospin preparations where cysts appeared unlabelled apart from occasional organisms which were associated with heavy localisations of deposit adjacent to the cyst walls. The significance of this is unknown but it may represent emerging trophozoites. Prolonged proteinase K digestion alleviated, but did not eliminate, this problem. This suggests that poor penetration of the relatively tough cysts may be at least partly responsible.

Since commencement of this study, genus- and subgenus-specific oligonucleotide probes for *Acanthamoeba*, based on small ribosomal subunit DNA have been described (Gast & Byers, 1995). These probes were designed to target all strains of the genus *Acanthamoeba* or subsets of strains identified by classical taxonomy or phylogenetic studies of small ribosomal subunit DNA sequences. The authors also describe the use of PCR-amplified DNA to increase the sensitivity of the method; pathological samples generally are very small samples with amoebae greatly outnumbered by human cells. A combination of a eukaryotic-specific primer (892C) and RGPG (designed to be specific for the entire genus *Acanthamoeba*) was used to prevent amplification of bacterial and human DNA respectively.

In the study by Gast γβεs (1995), probe specificity was tested by hybridisation to slot blots of amoeba genomic DNA and also compared with target sequences (53) determined in their laboratory. Discrepancies in the specificity of the probes for their intended targets were explained by sequence mismatches; indeed re-allocation of several species to a different group was suggested by the authors on this basis. None of these probes cross-reacted with *Hartmannella*. Two of the probes, including the one designed to be specific for the genus *Acanthamoeba*, did, however, cross-react with *Pseudomonas aeroginosa*, but not with any other bacteria or fungus tested. This cross-reactivity was eliminated by hybridising to PCR-amplified srDNA rather than genomic DNA.

In summary, further refinement of the ISH method for this application is required. The preliminary results described in this thesis and the slot blot results of Gast δβιεις (1995) do, however, offer hope for future development of probes for pan-amoeba and differential diagnosis of the 3 genera: this will only be possible when comparable genebank data for *Vahlkampfia* and *Hartmannella* are available to allow rational selection of probe sequences.

TABLE 4.3A

Cytopathic Effect on Keratocytes with Time:
Different Concentrations of *Acanthamoeba castellanii*

	Concentrations (AC/ml)						
	0	**10^1**	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**
3 hr	nc	nc	nc	nc	nc	+/-	+
6 hr	nc	nc	nc	nc	nc	+	++
9 hr	nc	nc	nc	nc	nc	++	+++
24 hr	nc	nc	nc	nc	+/-	++++	++++

TABLE 4.3B

Cytopathic Effect on Epithelial Cells with Time:
Different Concentrations of *Acanthamoeba castellanii*

	Concentrations (AC/ml)						
	0	**10^1**	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**
3 hr	nc	nc	nc	nc	nc	+/-	+/-
6 hr	nc	nc	nc	nc	nc	+/-	+/-
9 hr	nc	nc	nc	nc	nc	+	+
24 hr	nc	nc	nc	nc	nc	+	++

Code: nc: no damage apparent in epithelial cells
+/-: gaps appearing associated with cell shrinkage
+: 0-25% of monolayer destroyed
++: 25-50% of monolayer destroyed
+++: 50-75% of monolayer destroyed
++++: 75-100% of monolayer destroyed

TABLE 4.4

Image Analysis of Cytopathic Effect with Time:
***Acanthamoeba castellanii* on Keratocytes and Epithelial Cells**

	Proportion of Cells Remaining	
Time	**Keratocytes**	**Epithelial Cells**
3 hr	0.48	0.87
6 hr	0.55	0.84
9 hr	0.33	0.76
24 hr	0.07	0.49

The proportion of cells remaining, as measured by image analysis of area of blue remaining on Coomassie blue-stained coverslips, is expressed as a fraction of the relevant control cell type (with no amoebae added). The experiment was performed in triplicate and the results rounded to 2 decimal places.

TABLE 4.1

Cytopathic Effect on Keratocytes with Time:
Acanthamoeba, Vahlkampfia and Hartmannella

	Amoeba Genus (10^6/ml)		
	Acanthamoeba	***Vahlkampfia***	***Hartmannella***
3 hr	+	+/-	+/-
6 hr	++	+	+
9 hr	+++	++	++
24 hr	++++	++++	++++

Code: +/-: gaps appearing associated with cell shrinkage
+: 0-25% of monolayer destroyed
++: 25-50% of monolayer destroyed
+++: 50-75% of monolayer destroyed
++++: 75-100% of monolayer destroyed

TABLE 4.2

Image Analysis of Cytopathic Effect on Keratocytes with Time:
Acanthamoeba, Vahlkampfia and Hartmannella

Amoeba	Time	Proportion of Cells Remaining
AC_a	6 hr	0.23
V-EYE	6 hr	0.50
H-EYE	6 hr	0.41
AC_a	24 hr	0.03

The proportion of cells remaining, as measured by image analysis of area of blue remaining on Coomassie blue-stained coverslips, is expressed as a fraction of the relevant control cell type (with no amoebae added). The experiment was performed in triplicate and the results rounded to 2 decimal places.

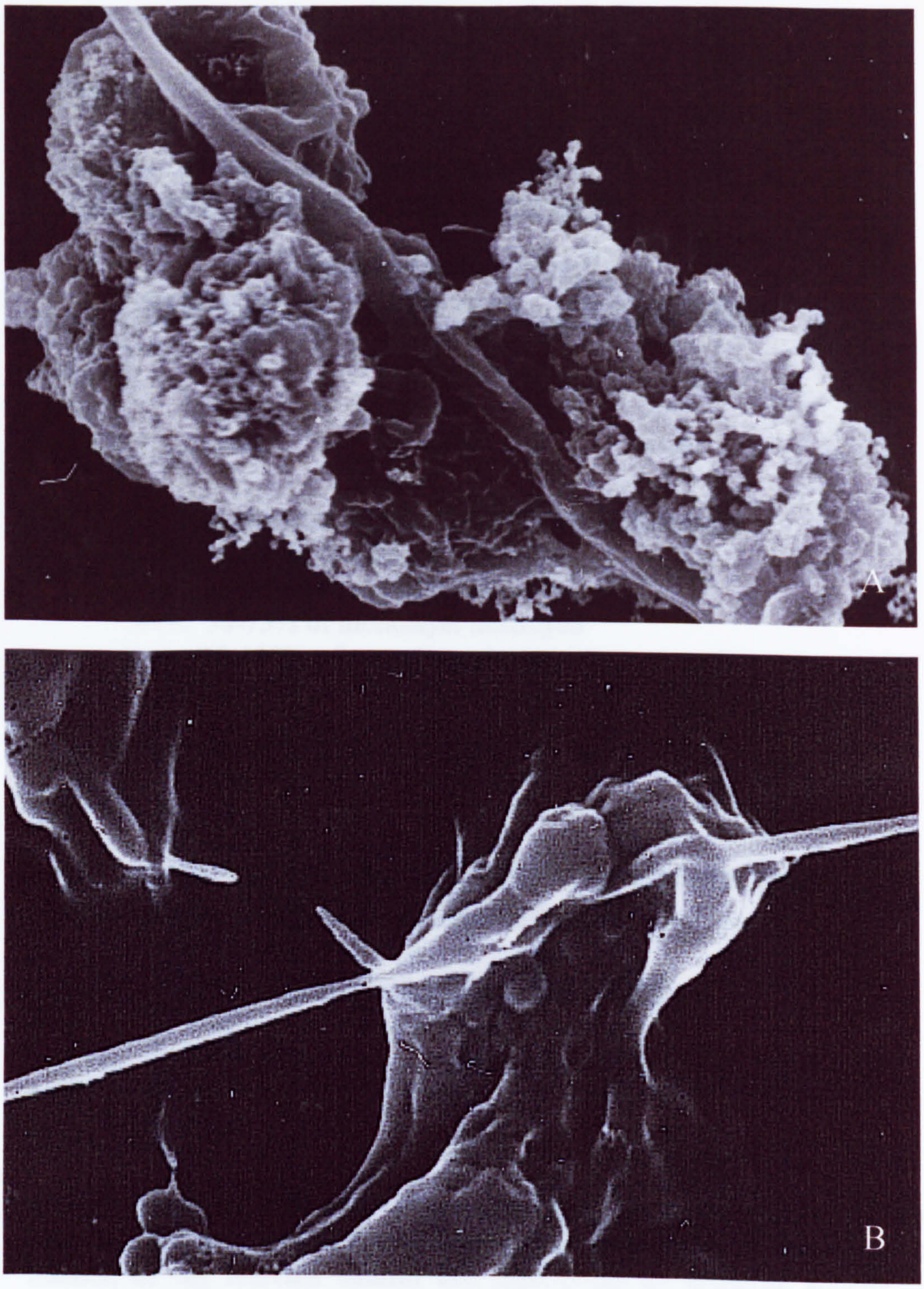
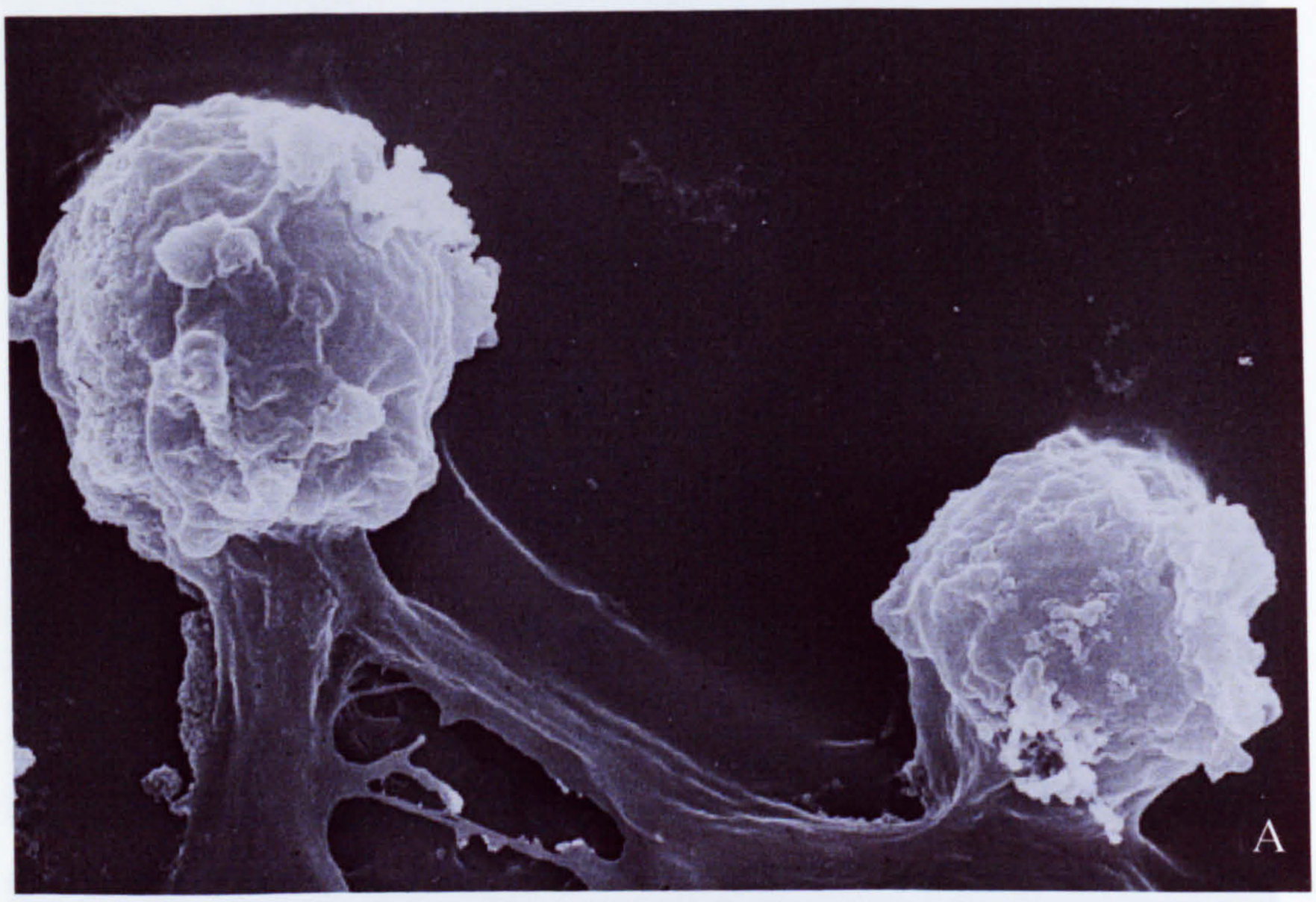


Fig 4.9 Scanning electron micrograph of cytopathic effects of amoebae on keratocytes

A) V-EYE trophozoite entwined around a keratocyte retraction process. (x11 000)

B) H-EYE trophozoite in intimate contact with a keratocyte retraction process. (x15 000)



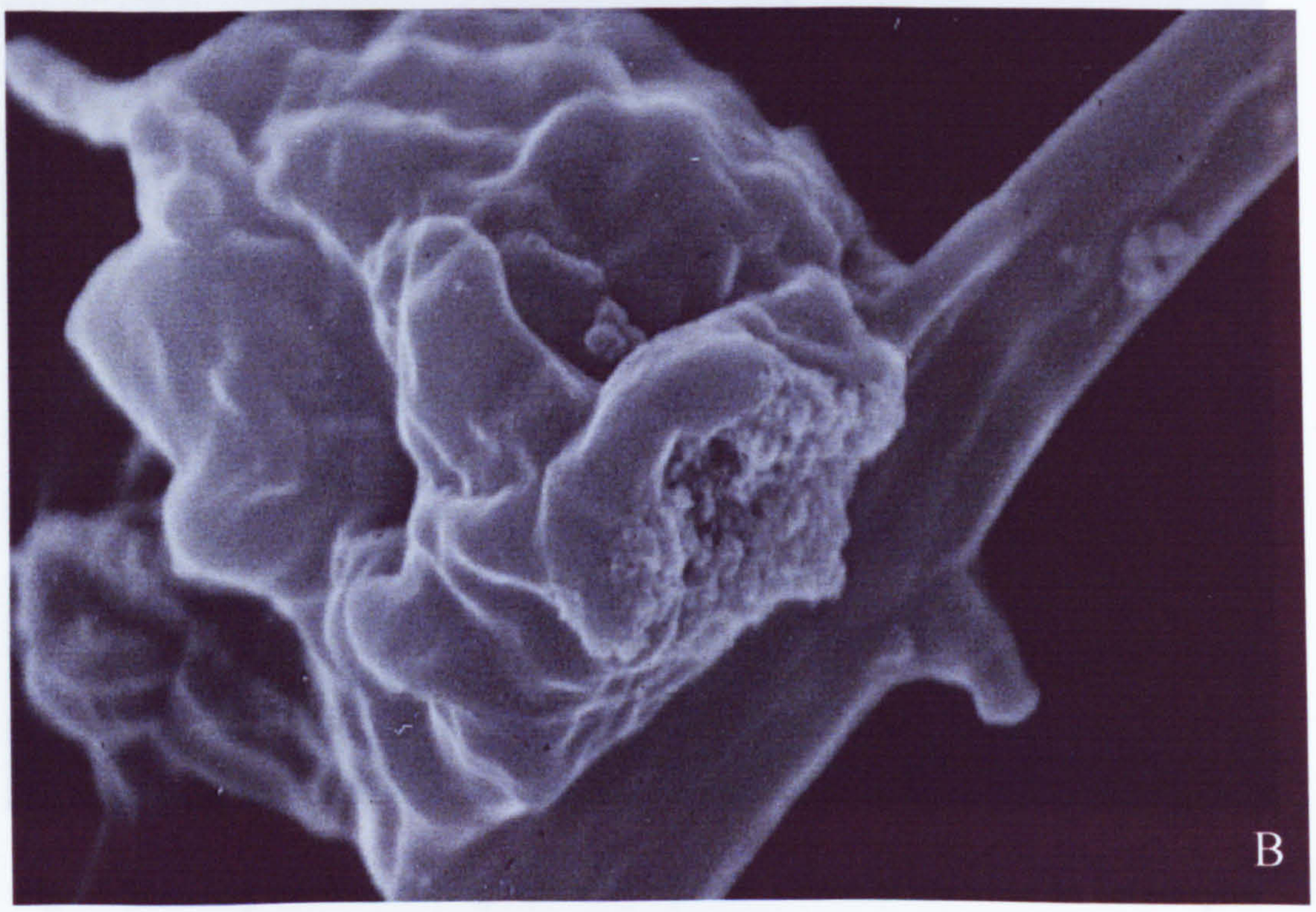


Fig 4.8 Scanning electron micrograph of cytopathic effects of amoebae on keratocytes

A) Two V-EYE trophozoites apparently attached to part of the membrane of a keratocyte. Note, however, that some shrinkage will have occured as a result of processing. (x8 000)

B) H-EYE trophozoite in contact with a keratocyte retraction fibre. (x20 000)

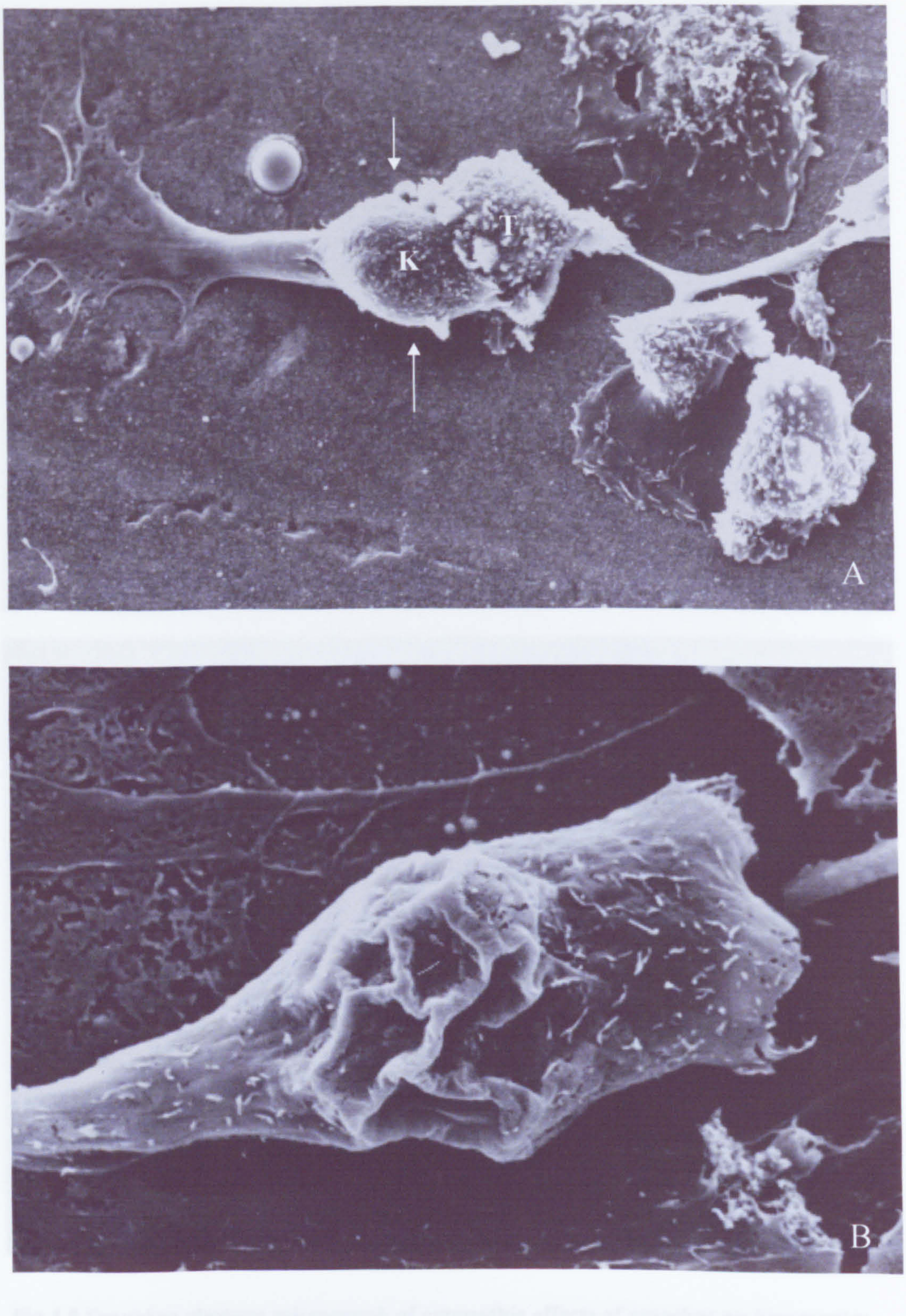
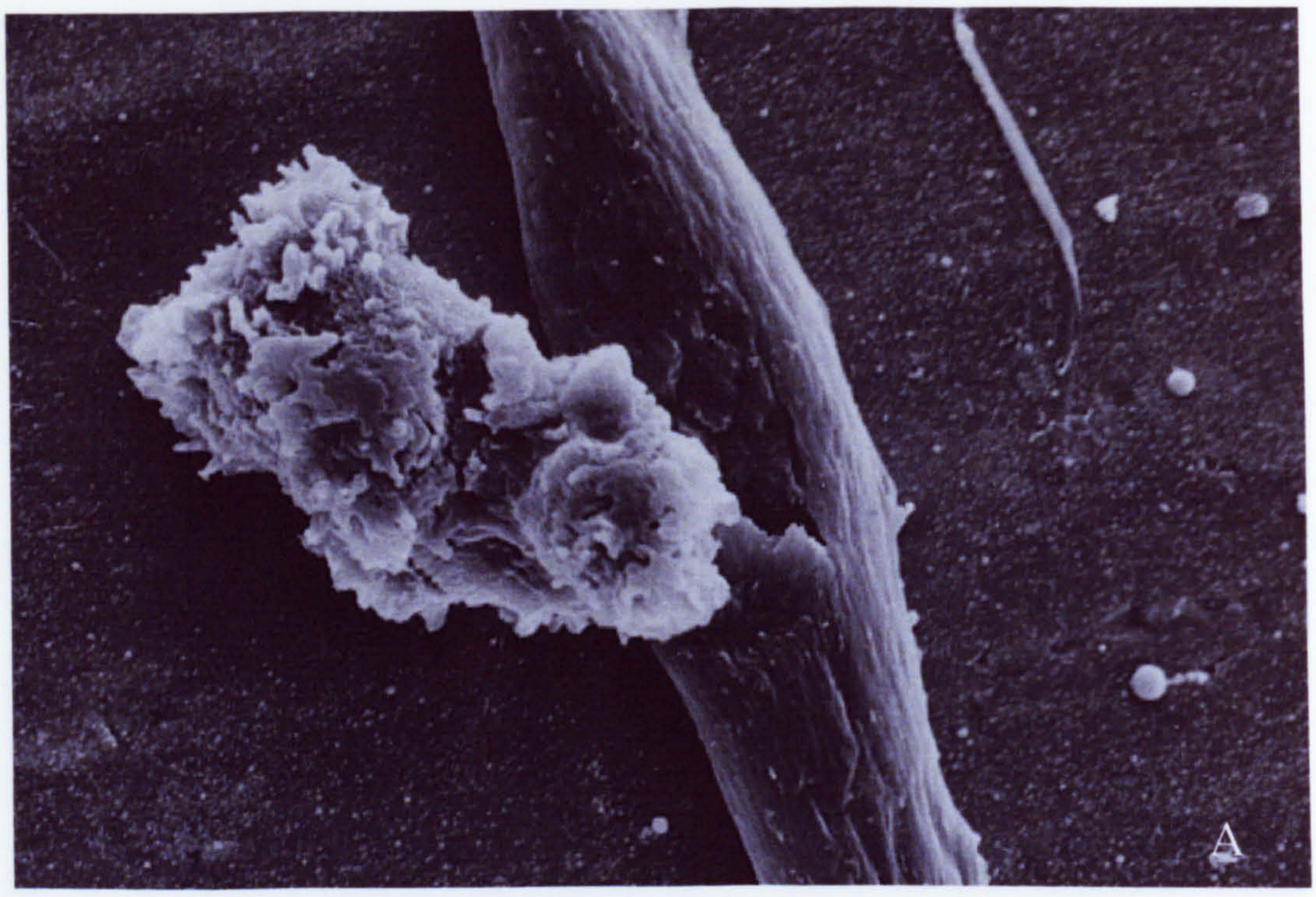


Fig 4.7 Scanning electron micrograph of cytopathic effects of amoebae on keratocytes

A) *Acanthamoeba castellanii* trophozoite (T) apparently within a damaged keratocyte (K). Note how the cell bulges around the organism (arrows). (x2 500)

B) *Acanthamoeba castellanii* cyst apparently within a damaged keratocyte. Note how the cell membrane follows the contour of the underlying cyst. (x4 000)



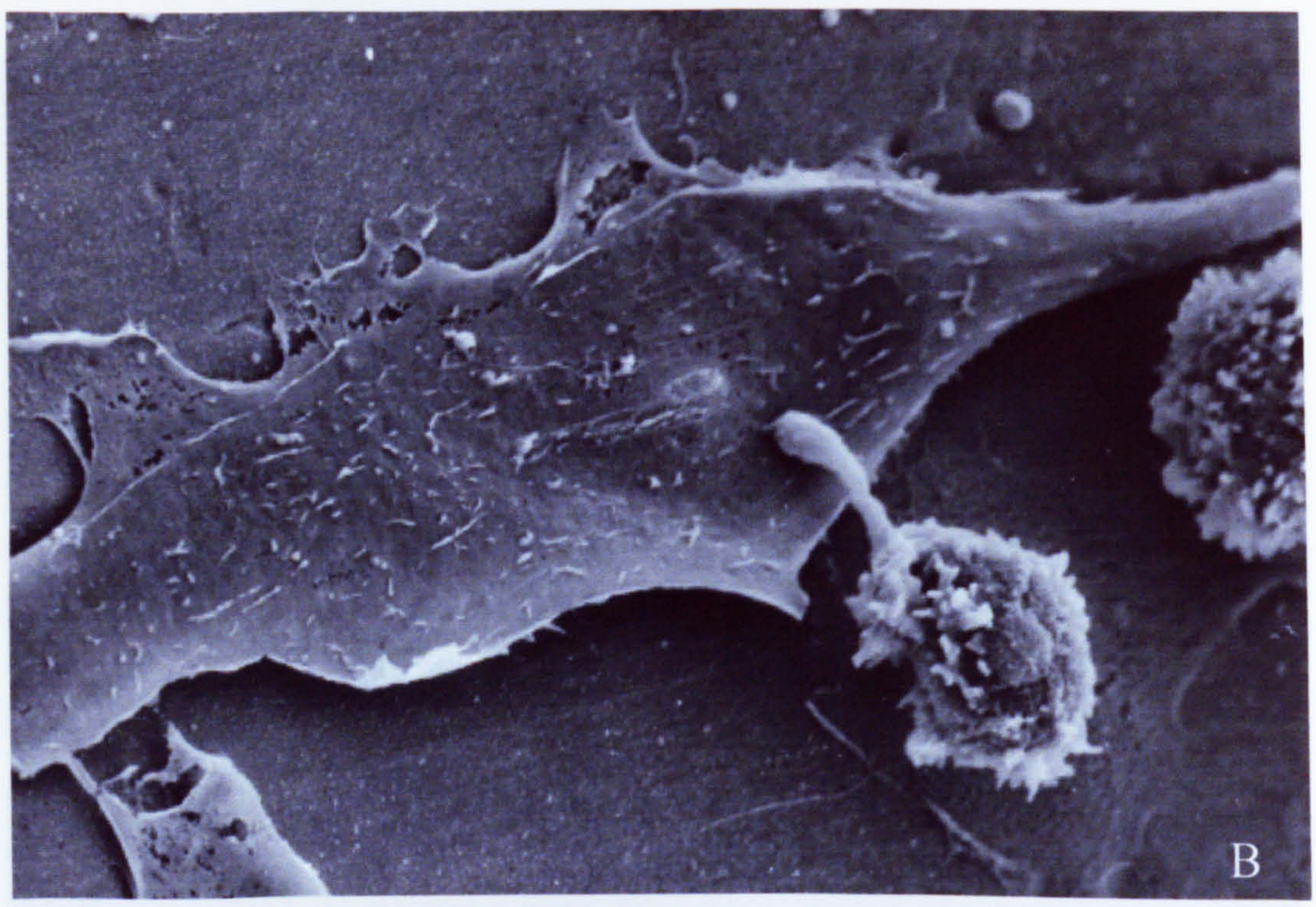


Fig 4.6 Scanning electron micrographs of cytopathic effect of amoebae on keratocytes

A) *Acanthamoeba castellanii* trophozoite apparently attacking the free edge of an area of damaged cell membrane. (x3 500)

B) *Acanthamoeba castellanii* cell process in contact with a keratocyte. (x2 600)

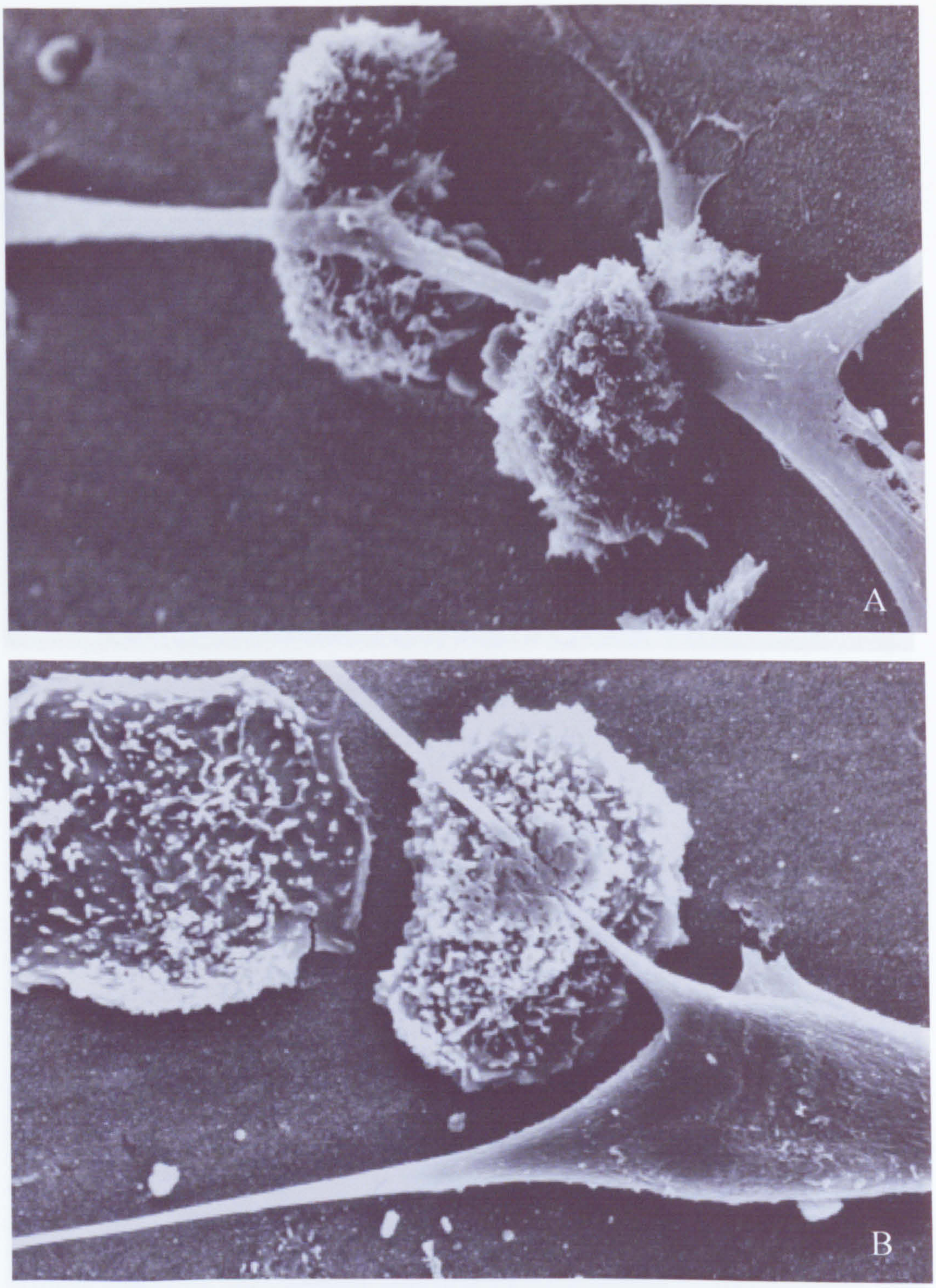


Fig 4.5 Scanning electron micrograph of cytopathic effects of amoebae on keratocytes

A) *Acanthamoeba castellanii* trophozoites passing underneath and around a keratocyte process which has become detached from the substratum. (x2 400)

B) *Acanthamoeba castellani* trophozoite in intimate contact with a retraction fibre of a keratocyte. (x2 800)

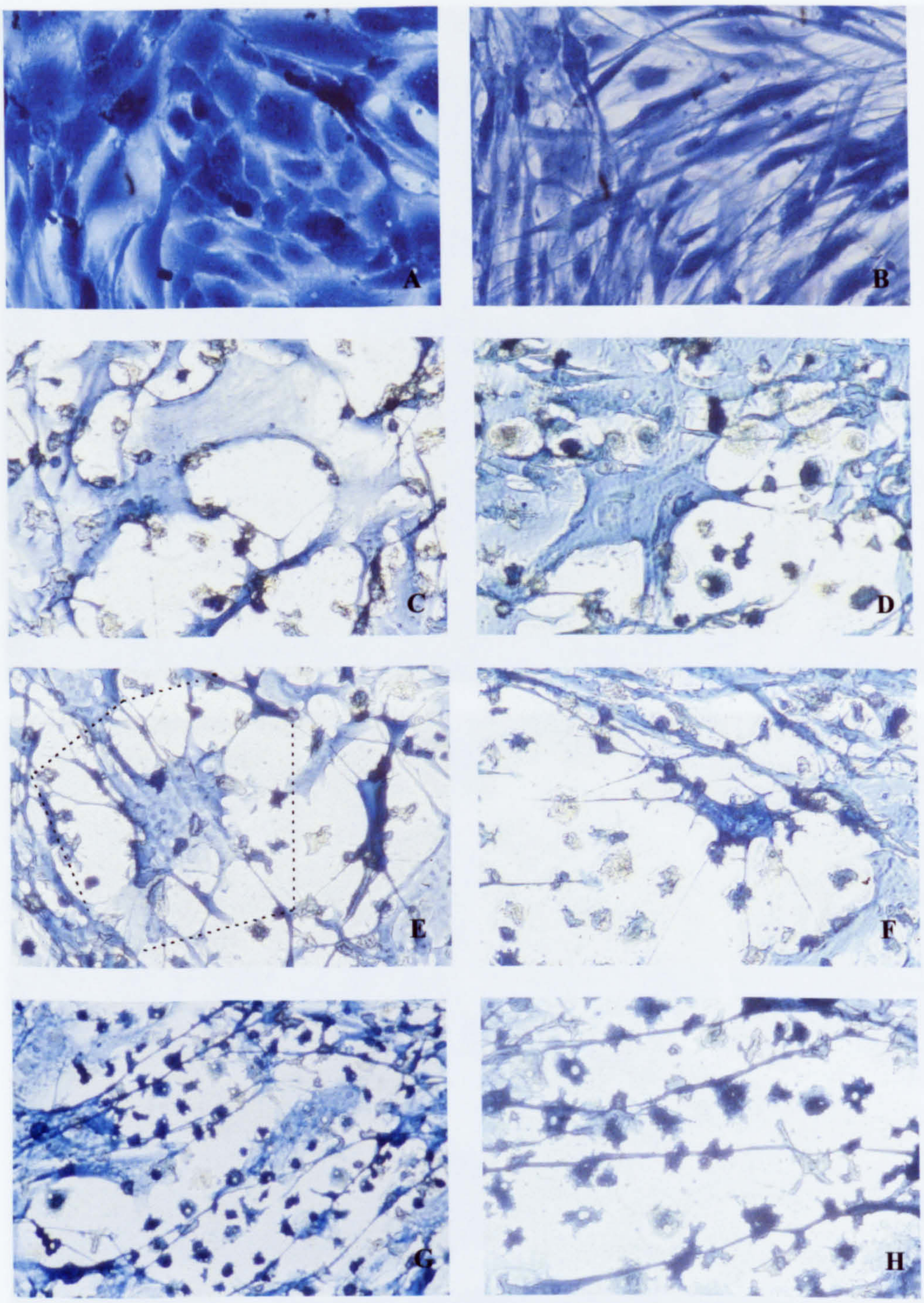
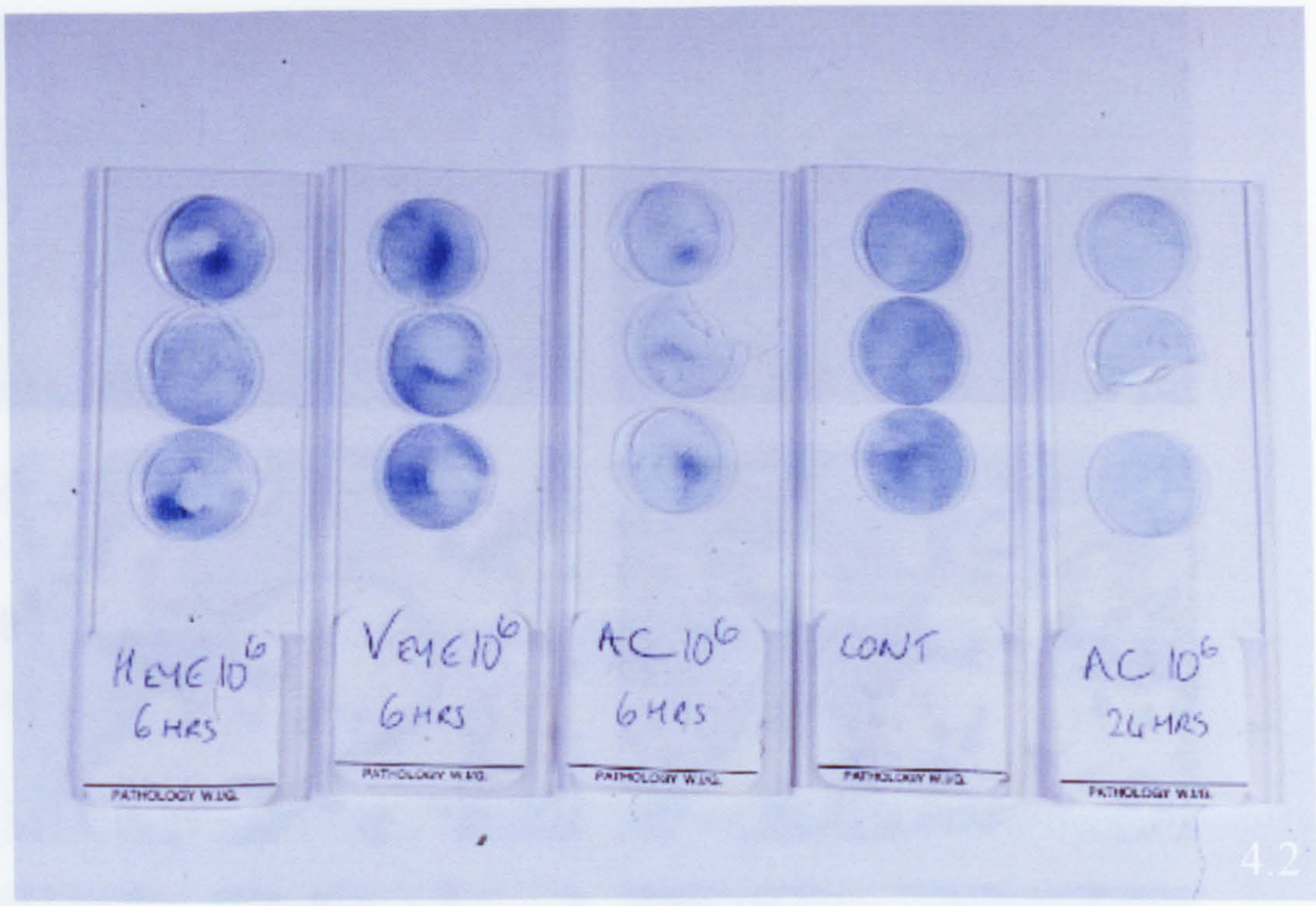


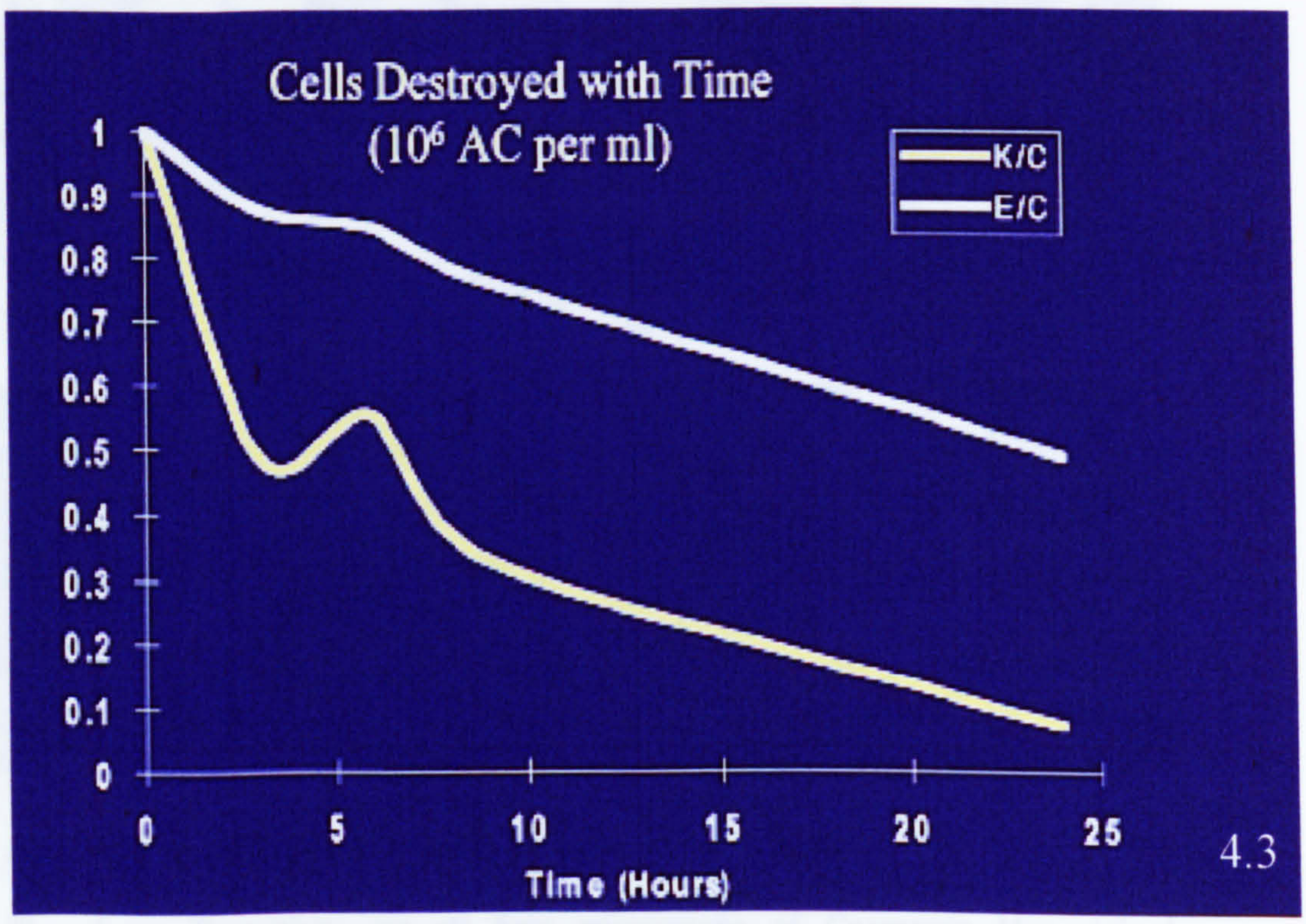
Fig 4.4 Cytopathic effect on epithelial cells and keratocytes.

The above illustrations are all light micrographs of Coomassie blue stained preparations demonstrating the cytopathic effect of *Acanthamoeba castellanii* (10^6/ml) on both cultured epithelial cells and keratocytes.

A) Typical 'crazy-paving' appearance of epithelial monolayer. (x360)
B) Typical chaotic growth pattern of keratocytes. (x360)
C) Epithelial cell being attacked around the cell margins by trophozoites. (x580)
D) Epithelial cell demonstrating early cell shrinkage secondary to detachment from substratum. (x580)
E) Epithelial cell demonstrating more advanced loss of adhesion, but still attached to adjacent cells by retraction fibres (dotted line - presumptive previous area: x580).
F) Markedly shrunken epithelial cell with retraction fibres. (x580)
G) Shrunken keratocytes with retraction fibres. (x360)
H) Amoebae trophozoites associated with keratocyte retraction fibres. (x580)



4.2



4.3

Fig 4.2 Coomassie blue slide preparations for image analysis

These slides represent a comparison of the relative pathogenicity of *Acanthamoeba, Vahlkampfia* and *Hartmannella* for keratocytes. Similar slides were prepared for image analysis for comparison of the relative susceptibility of epithelial cells and keratocytes to *Acanthamoeba castellanii.*

Fig 4.3 Graph of relative susceptiblity of epithelial cells and keratocytes to *Acanthamoeba castellanii*.

The figures on the y-axis represent the proportion of cells remaining: the area of cells remaining is expressed as a fraction of the area of cells on the relevant control (with no amoebae added). Code: K-keratocytes; E- epithelial cells; C-control)

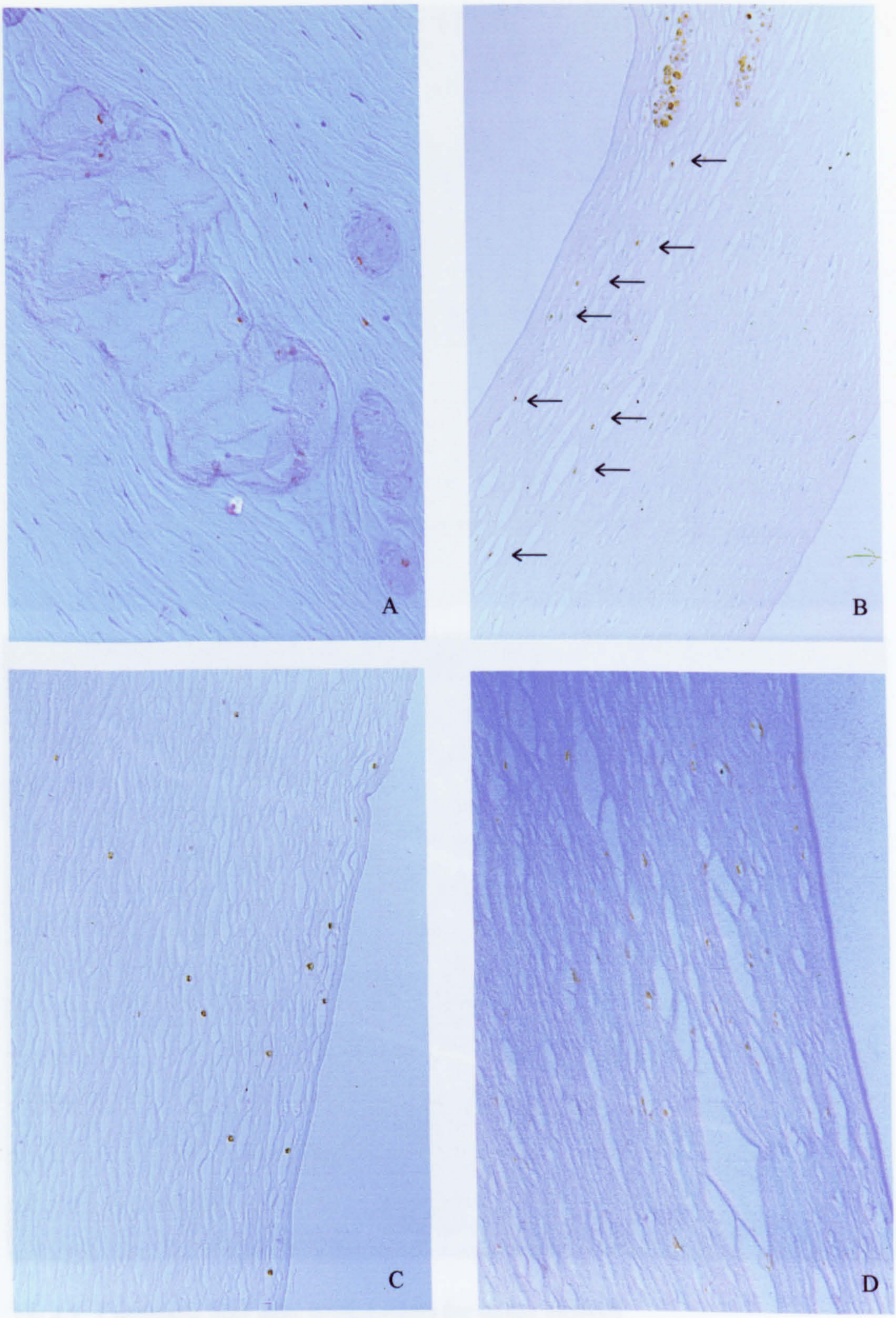


Fig 4.1 Investigation of the pathogenicity of the 3 genera of amoebae for corneal tissue

A) An early attempt at inoculation of amoebae into corneal tissue, which is tough and collagenous, with marked tissue disruption and few amoebae (see section 2.1.2.2).
B) A later, more skilled example, demonstrating migration of *Acanthamoeba castellanii* trophozoites (arrows) from the site of inoculation within 24 hrs.
C) Seven days post-inoculation, keratocytes are depleted and the trophozoites have encysted.
D) Invasion of V-EYE at 24 hr, after co-incubation with a whole cornea as described in section 2.3.1.1.

(Light microscopy: SK anti-*Acanthamoeba* ABC-Peroxidase)
(A&D - mag x140: B&C - mag x70)

4. PATHOGENICITY OF *ACANTHAMOEBA*, *VAHLKAMPFIA* AND *HARTMANNELLA*

4.1 Introduction

4.1.1 Pathogenicity for Corneal Tissue in Organ Culture

This chapter describes the development of a model for invasion of human corneal tissue by *Acanthamoeba castellanii*. Using this definitive model the resulting pathology with this organism is then detailed. The ability of *Vahlkampfia* and *Hartmannella* to invade and produce stromal infection is also tested.

The behaviour of *Acanthamoeba castellanii* within human corneal tissue, and the resulting pathology, was also assessed post-inoculation of the organism into corneal stroma.

4.1.2 Cytopathogenicity for Corneal Cells in Tissue Culture

The pattern of cell damage produced on primary cultures of keratocytes when co-incubation was continued for up to 10 days is described for *Acanthamoeba castellanii* at a range of concentrations. Evidence of the potential pathogenicity of V-EYE and H-EYE was provided by quantitative comparison of the relative ability of the 3 genera of amoebae to produce a cytopathic effect on keratocytes as recorded over a 24 hr incubation period. Comparison of the relative susceptibility of epithelial cells and keratocytes to such cell damage is also detailed for a 24 hr co-incubation with various concentrations of *Acanthamoeba castellanii*.

Investigation of the mechanism of cell damage involved in production of a cytopathic effect by representatives of each of the 3 genera of amoebae is described utilising time lapse video, light and scanning electron microscopy

(SEM) studies. In order to clarify the importance of mechanical factors compared to secretory factors, investigation of the ability to produce a similar cytopathic effect with conditioned medium, or in the absence of physical contact between cells and amoebae, is outlined. The role of apoptosis in the death of cells exposed to *Acanthamoeba castellanii in vitro* is also assessed.

4.2 MATERIALS AND METHODS

4.2.1 Pathogenicity for Corneal Tissue in Organ Culture

4.2.1.1 Invasion studies

Various methods, described in detail in Section 2.3.1.1, were developed to attempt to assess the ability of the 3 genera of amoebae to invade corneal tissue. Preliminary experiments, all with *Acanthamoeba castellanii*, utilised rabbit corneas while later studies were performed with human corneas; in total 10 eyebank corneas were required for this section of the study. The 'definitive' method finally developed involved incubation of whole corneas inverted (i.e. epithelium down) on a contact lens supported in a contact lens holder (Fig 2.3B). The resulting pathology after 24 hours and after 10 days co-incubation is described for *Acanthamoeba castellanii*. This method was then used to assess the invasiveness of V-EYE and H-EYE. The presence of amoebae within the tissue was detected by immunolabelling with SK anti-*Acanthamoeba* antiserum by the ABC-Peroxidase technique (Appendix C).

4.2.1.2 Post-inoculation studies

The behaviour of *Acanthamoeba castellanii* within corneal stroma was also assessed post-inoculation. As described in Section 2.3.1.2, preliminary studies had been performed to optimise the technique, both with regard to inoculation method and fixation, in order to maximise preservation of tissue architecture. These initial experiments also suggested that the trophozoites could migrate throughout the tissue from the site of inoculation within 24 hr; verification of this merited incubation times of 3, 6, 9 and 24 hr. Incubation times of 1, 2, 3, 4,

5, 6, 7 and 10 days were adopted to investigate the ongoing behaviour of the organism with respect to migration, disruption of stromal lamellae, keratocyte depletion and encystment. All these studies were conducted following inoculation of >95% trophozoites of axenic *Acanthamoeba castellanii*. The ability of the organism to excyst in corneal tissue, however, was assessed post-inoculation of 95% cysts after incubation times of 1, 2, 3 and 6 days. Discounting the preliminary experiments, 12 separate inoculations were performed. Amoebae within the tissue were located by immunolabelling with SK anti-*Acanthamoeba* antiserum using the ABC-Peroxidase technique (Appendix C). The potential for use of the core biopsy technique (Fig 2.4 & Section 2.3.1.2) to preserve pathological specimens was also assessed.

4.2.2 Cytopathogenicity for Corneal Cells in Tissue Culture

4.2.2.1 Time/concentration study of the cytopathic effect of Acanthamoeba *on keratocytes*

Preliminary experiments investigated the time course of the cytopathic effect produced by different concentrations of *Acanthamoeba castellanii* (10^1, 10^2, 10^3, 10^4, 10^5 and 10^6/ml/well) on cultured keratocytes plated out overnight at a concentration of 50 000/ml/well (Section 2.3.2.1). Daily observation of the effects of co-incubation were conducted for 10 days with regard to both the corneal cells and the amoebae. The experiment was performed in triplicate with inclusion of a negative control of keratocytes in EGM 2% only in each case.

4.2.2.2 Comparison of the cytopathic effects of Acanthamoeba, Vahlkampfia *and* Hartmannella *on keratocytes*

Prior to comparison of the 3 different genera, the ability of *Acanthamoeba castellanii* trophozoites in axenic culture (AC_a) to produce a cytopathic effect was compared with that of *Acanthamoeba castellanii* trophozoites cultured in 25 cm^2 flasks containing heat killed bacteria (AC_b). Cultures of AC_a and AC_b (10^6/well in the log phase of growth) were co-incubated with keratocytes (50 000/ml/well) for 24 hr.

Following this assessment of the effect of axenisation on the virulence of *Acanthamoeba castellanii*, the species selected for comparison were axenic *Acanthamoeba castellanii* trophozoites and monoxenic V-EYE and H-EYE trophozoites, all in the log phase of growth and all at a concentration of 10^6/ml/well. Preliminary experiments involved visual estimation of the proportion of cells remaining at 3, 6, 9 and 24 hr for each of the 3 representative strains. On the basis of these results, in order to maximise any potential differences, 6 hr of incubation was selected for formal quantitative analysis. Triplicate sets of coverslips were fixed following 6 hr of incubation with each of the 3 genera. Following Coomassie blue staining the results were assessed by image analysis (Section 2.3.2.2). A negative control of keratocytes in EGM 2% only, in triplicate, was also included.

4.2.2.3 Comparison of the susceptibility of epithelial cells and keratocytes to Acanthamoeba

Similarly, the relative susceptibility of confluent epithelial cells and keratocytes (50 000/ml/well) to the cytopathic effect produced by *Acanthamoeba castellanii* was also investigated. Preliminary experiments utilised *Acanthamoeba castellanii* at concentrations of 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6/ml along with a negative control of EGM 2% only for each corneal cell type (Section 2.3.2.1). The proportion of cells remaining after 24 hr for each concentration of amoeba trophozoites was estimated by eye and recorded.

More detailed time studies investigated the effects of *Acanthamoeba castellanii* trophozoites at a concentration of 10^6/ml/well only; coverslips were removed for formalin fixation at 3, 6, 9 and 24 hr. A negative control, fixed at 24 hr, which had not been exposed to *Acanthamoeba castellanii,* was included for both epithelial cells and keratocytes. The experiment was performed in triplicate for each time interval. Again the results were compared by image analysis of Coomassie blue stained coverslips (Section 2.3.2.2).

4.2.3 Mechanism of Cytopathogenicity

4.2.3.1 Production of cytopathic effects in the presence of physical contact

Studies to elucidate the mechanism of production of the observed cytopathic effect included time lapse video microscopy in combination with light microscopy and SEM (Section 2.3.3.1). These were all performed on keratocytes (50 000/ml/well) exposed to either axenically grown *Acanthamoeba castellanii* trophozoites or monoxenically grown V-EYE or H-EYE trophozoites. The same methods were also applied to confluent epithelial cells exposed to *Acanthamoeba castellanii* only.

4.2.3.2 Production of cytopathic products

Preliminary experiments involved medium conditioned simply by exposing keratocytes to *Acanthamoeba castellanii*. Various attempts were also made to produce conditioned medium through release of intracellular contents (Section 2.3.3.2). Finally, prolonged (1-2 hr) manual grinding of trophozoites in a homogenisor demonstrated disruption of significant numbers of trophozoites. The medium thus produced from *Acanthamoeba castellanii* trophozoites at an original concentration of both 10^6/ml and 10^7/ml in EGM 2% was added to wells of keratocytes (50 000/ml/well) which were then incubated as before, with daily observation for up to 10 days.

As the above method was rather labour intensive, the ability of the cells to produce cell damage in the absence of physical contact was also investigated using transwells (Costar Ltd). This apparatus is a double welled incubation system consisting of a 24 well plate with separate mesh-bottomed inserts (Fig 2.5). Axenic *Acanthamoeba castellanii* and monoxenic V-EYE and H-EYE trophozoite, all in the log phase of growth at a concentration equivalent to 10^6/ml, were variously co-incubated with keratocytes in the transwell plates, with daily observation of the keratocytes for up to 10 days (Section 2.3.3.2).

Following formalin fixation, the presence of apoptotic cells amongst cells that had been damaged by *Acanthamoeba castellanii* in the transwell apparatus or by the contents of lysed amoebae was then tested for using the Oncor Apoptaq™ *in situ* hybridisation apoptosis detection kit (Section 2.3.3.2).

4.3 Results

4.3.1 Pathogenicity for Corneal Tissue in Organ Culture

4.3.1.1 Invasion studies

In this study it was not possible to demonstrate invasion of the epithelium by *Acanthamoeba castellanii* in the preliminary experiments with rabbit corneas. Hence the utilisation of a range of methods of incubation for human corneas (Section 2.3.1.1) in an attempt to establish intrastromal infection with *Acanthamoeba castellanii*. With human corneas, however, invasion was successfully demonstrated following incubation with *Acanthamoeba castellanii* (10^6/ml) within 24 hr in all cases (i.e. for strips of cornea and for whole or scarred corneas whether epithelium or endothelium uppermost); by day 10 most trophozoites had encysted. Aside from depletion of keratocytes with time, there was no other significant abnormality. Using the definitive method of a whole, unscarred cornea incubated epithelium-down on a contact lens, stromal invasion was also demonstrated with both V-EYE and H-EYE following 24 hr incubation (FIG 4.1D).

4.3.1.2 Post-inoculation studies

The inoculation technique (Section 2.3.1.2 & Fig 2.3A) required some practice to enable successful instillation of organisms with minimal disruption of tissue architecture (Fig 4.1A & B). Within 24 hr trophozoites of *Acanthamoeba castellanii* had migrated throughout the tissue from the site of inoculation (Fig 4.1B). Further incubation resulted in decreasing numbers of keratocytes with time, with encystment of trophozoites occurring in line with keratocyte

depletion. Apart from the actual site of inoculation, relatively little disruption of tissue architecture was observed (Fig 4.1B). Different inoculations followed slightly different time courses, but generally some cysts had appeared by day 3 or 4, with the majority of keratocytes destroyed by day 5-7 and complete encystment by day 10 (Fig 4.1C). Alternatively, following inoculation of >95% cysts, excystment and migration throughout the tissue had occurred by 24 hr. These studies were performed on 5 μm sections cut from the whole block of paraffin embedded tissue. It was, however, also demonstrated that use of the renal biopsy needle to take a core biopsy of such tissue blocks enabled serial sections to be cut through areas of interest, without destroying the whole of the tissue.

4.3.2 Pathogenicity for Corneal Cells in Tissue Culture

4.3.2.1 Time/concentration study of the cytopathic effect of Acanthamoeba *on keratocytes*

The time course of the effects of incubating different concentrations of *Acanthamoeba castellanii* on keratocytes for a 10 day period is summarised below. At a concentration of 10^6/ml the trophozoites were able to produce destruction of the majority (>75 %) of the 'monolayer' within 24 hr. Further destruction of the remaining cells occurred over the following days but this was much less marked than the initial period of rapid destruction. Rounding up and/or encystment occurred at approximately day 5-7 in the face of dwindling keratocyte numbers; the cytopathic effect was never observed to continue to complete destruction of the 'monolayer'.

At a concentration of 10^5/ml a similar time course was followed although the effects were less marked both as regards the initial period of rapid destruction in the first 24 hr and also the slower accumulation of cell damage occurring over the following days. As frank cell destruction ceased, the amoebae appeared to be rounded up, rather than roaming actively; whether these represented immature cyst forms is unknown but few mature, double walled

cysts were observed.

At a concentration of 10^4/ml the trophozoites did produce some initial evidence of cell damage; this may mainly have been caused by cell shrinkage rather than actual destruction (Section 4.3.3.1). Over the following days, however, such gaps did not advance to larger areas of cell destruction; indeed the gaps tended to disappear, with restoration of a healthy-appearing 'monolayer' and rounding-up/encysting of the amoebae. This appeared to represent a cut-off point as concentrations below 10^4/ml produced no demonstrable cell damage; the trophozoites were observed to mill about over the keratocytes for 2-3 days then simply rounded-up/encysted in spite of the presence of a plentiful food source.

4.3.2.2 Comparison of the cytopathic effects of Acanthamoeba, Vahlkampfia *and* Hartmannella *on keratocytes*

Comparison of the relative ability of AC_a and AC_b to produce a cytopathic effect demonstrated that, in both cases, near complete destruction of the monolayer was achieved by 24 hr. V-EYE and H-EYE also produced significant cytopathic effect on keratocytes within 24 hr at a concentration of 10^6/ml/well, with near destruction of the monolayer. Table 4.1 demonstrates the progress of cell destruction with time, as assessed by eye. Subjectively it appeared that, although the degree of destruction was similar after 24 hr of co-incubation, initial destruction was more rapid for AC_a as compared to monoxenic V-EYE and H-EYE. Thus, in order to maximise any difference, the proportion of cells surviving after co-incubation with *Acanthamoeba castellanii*, V-EYE and H-EYE was recorded after only 6 hr. The results were assessed by image analysis of Coomassie blue stained coverslips (Fig 4.2), and are recorded for each species in Table 4.2. The figures represent the area of blue remaining expressed as a proportion of the control, which consisted of keratocytes only.

4.3.2.3 Comparison of the susceptibility of epithelial cells and keratocytes to **Acanthamoeba**

Results of the preliminary experiments measuring the effect of different concentrations of *Acanthamoeba castellanii* on epithelial cells and keratocytes are summarised in Table 4.3A & B. As described above (Section 4.3.2.1) a cut-off point could be demonstrated, in this case for both types of cells: for epithelial cells this was 10^5/ml/well while for keratocytes it was 10^4/ml/well, although the damage produced at these concentrations was slight. Concentrations of amoebae below these respective levels had no demonstrable effect. The cytopathic effect produced by trophozoites at a concentration of 10^5/ml/well, and more particularly at 10^6/ml/well, was much more marked for keratocytes than for epithelial cells. Epithelial cells and keratocytes, however, had both suffered significant damage by 24 hr of incubation in the presence of *Acanthamoeba castellanii* at 10^6/ml/well; this concentration was therefore selected for more detailed studies.

As a measure of surviving cells, the area of blue remaining with time, as determined by image analysis, is recorded for each of the 2 types of corneal cells in Table 4.4. By expressing the results for each time interval as a fraction of the control value, the loss of cells with time can be more clearly demonstrated for both epithelial cells and keratocytes (see graph in Fig 4.3). Clearly keratocytes are more susceptible to cell damage and destruction by *Acanthamoeba castellanii*, as the area of cells remaining at 24 hr, expressed as a fraction of the relevant control value, is less.

Closer inspection of the results, however, reveals that the rate of loss of cells for both keratocytes and epithelial cells, when compared to their respective controls, is, in fact, the same in the later stages of the 24 hr experimental period (i.e. after 9 hr). The initial rate of loss of keratocytes is, however, much more rapid than that observed for epithelial cells (i.e. the graph is steeper). This is true as regards at least the first 3 hr, whether the peak observed at 6 hr is regarded as artefact or a real phenomenon, or, indeed, even if the 3 hr value is

considered artificially low (see discussion - Section 4.4.2.3).

4.3.3 Mechanism of Cytopathogenicity

4.3.3.1 Production of cytopathic effects in the presence of physical contact

Time lapse video microscopy (see CD-ROM) gave a dynamic record of the ongoing process: it also facilitated interpretation of the still images achieved by light microscopy and also by SEM (see below). For representatives of each of the 3 genera of amoebae it revealed how the observable cytopathic effect proceeded with time, although speeded up to 80X real time. Initially trophozoites were observed to roam about actively over the surface of the cells, apparently at random. They also passed between the cells; this phenomenon occurred later, but was also particularly obvious, with epithelial cell monolayers. Amoebae even frequently passed underneath cells, in effect mechanically weakening the anchoring system of the cells; this was more marked in keratocyte cultures but was also observed at an advanced stage with cultured epithelial cells.

With regard to both cell types, the first demonstrable change in the cells was that they shrunk away from the underlying substratum, leaving behind retraction fibres (Fig 4.4) and later rounding up as they detached completely. Once this occurred the movements of the amoebae appeared less random and were more directed towards the cells than the intervening spaces that had developed. Movement of trophozoites under, over and around the retraction fibres was also observed. These vulnerable processes were frequently disrupted; this presumably led to extrusion of cytoplasmic contents. Whether this occurred as a result of mechanical traction forces or was due to a snipping-type action of amoebae could not be clearly distinguished.

Minor damage to the cells appeared to render them more susceptible to further damage. At this stage trophozoites were able to insert pseudopodia and even to

enter into partially disrupted cells. They appeared to phagocytose remnants of cells and to attack relatively intact cells by trogocytosis. In this manner the gaps which had initially appeared due to cell shrinkage were enlarged by actual destruction of individual cells.

Still images by light and scanning electron microscopy substantiated the above description of the sequence of events for production of cytopathic effects. Low power images of the ongoing process yielded the impression that the amoebae were concentrated around individual cells or groups of cells rather than randomly migrating around the intervening regions (Fig 4.4). Cells with retraction fibres indicating the prior point of contact were present in both keratocytes and epithelial cell cultures; in the latter the retraction fibres were particularly obvious as the cells had previously formed a complete monolayer (Fig 4.4). High power views by SEM also demonstrated trophozoites beneath and also wrapped around such retraction fibres (Fig 4.5). Once the cells were damaged they were attacked by trogocytosis (Fig 4.6A). Finger-like projections were also observed in contact with cells (Fig 4.6B). Amoebae within cells were observed both as trophozoites and also as cysts (Fig 4.7). Similar processes were observed for V-EYE and H-EYE (Figs 4.8 & 4.9). No such studies of the cytopathogenicity of *Vahlkampfia* or *Hartmannella* have been described.

4.3.3.2 Production of cytopathic products

Medium conditioned simply by co-incubating *Acanthamoeba castellanii* and keratocytes for up to a week failed to produce a cytopathic effect on fresh keratocytes, even with incubation times of up to 10 days. Damage to cells did, however, occur with cell-free parasite lysates of *Acanthamoeba castellanii* and using the transwell apparatus. In both these cases, production of a cytopathic effect required significantly longer than when the amoebae and cells were in direct contact. Ultimately, however, the destruction produced was just as complete when co-incubation was sufficiently prolonged. Damage and destruction of cells produced in this experiment was also subjectively more

uniformly distributed than either that produced by amoebae in transwell apparatus or by amoebae in direct contact with the cells. Cell damage observed with both experimental methods consisted of increased granularity of cells with vacuolation and also cell shrinkage. This was followed by rounding up, detachment and apparent disintegration. The same observations regarding cell appearance and the rapidity of the effects were also true for cells exposed to both V-EYE and H-EYE in the transwell apparatus.

For the purpose of examination for apoptotic cells, cells exposed to *Acanthamoeba castellanii* were generally fixed between day 7-10 for transwell experiments, and at day 2 for cells exposed to medium conditioned with mechanically disrupted amoebae. The transwells exhibited marked damage to the cells: medium conditioned with 10^7 amoebae/ml had also produced destruction of >75% of the cells while cells exposed to medium conditioned with 10^6 amoebae/ml exhibited evidence of cell damage but little actual cell destruction.

Apoptotic cells were not demonstrated amongst cells damaged by medium conditioned by *Acanthamoeba castellanii*, nor amongst cells damaged by the same species in the transwell apparatus. A very occasional equivocally positive cell was detected, but they represented no more than the odd cell that might be expected to be undergoing programmed cell death in any population of cells. They were also present in the negative control that had not been exposed to *Acanthamoeba*. They certainly could not alone have accounted for the loss of cells observed. In contrast, the Kit control for the procedure exhibited large numbers of strongly labelled cells.

4.4 Discussion

4.4.1 Pathogenicity for Corneal Tissue in Organ Culture

4.4.1.1 Invasion studies

Invasion by *Acanthamoeba castellanii* was not successfully demonstrated in the preliminary experiments with rabbit corneas despite the fact that later experiments involving incubation of human corneas under similar conditions did produce a positive result. I am unaware of any literature recording successful invasion of rabbit cornea by *Acanthamoeba*. Indeed Niederkorn *et al.* (1992) not only failed to demonstrate invasion but also record that *Acanthamoeba* fail to even bind to rabbit corneas in significant numbers. As binding may be considered a prerequisite for invasion (Moore *et al.*, 1991; Ubelaker *et al.*, 1991), this failure may underlie the inability to establish *Acanthamoeba* keratitis in the rabbit (He *et al.*, 1992) when intrastromal inoculation was successful (Font *et al.*, 1981 & 1982; Cote *et al.*, 1991). Other conflicting reports, however, suggest that *Acanthamoeba castellanii* can in fact bind to rabbit corneal epithelium in organ culture (Panjwani *et al.*, 1997) and indeed to glycolipids extracted from cultured rabbit corneal epithelial cells (Panjwani *et al.*, 1992). *Acanthamoeba polyphaga* and *Acanthamoeba culbertsoni* have also been shown to bind to cultured rabbit corneal epithelial cells (Morton *et al.*, 1991a&b).

Furthermore, failure to demonstrate invasion of rabbit corneas was in spite of the fact that incubation of such a relatively large cornea in the individual well of a 24 well plate involved either distortion of the epithelial architecture (if the scleral rim was left attached) or, alternatively, exposure of a cut edge of cornea (if a button was trephined to fit). In contrast, invasion of human cornea by *Acanthamoeba castellanii* was successfully achieved by all methods tested.

Indeed, quite the opposite result might have been expected due to the additional barrier to penetration present in the human cornea in the form of

Bowman's layer, which is not present in rabbit corneas. This was the rationale behind the use of scarred corneas. The results, however, demonstrated that penetration of *Acanthamoeba castellanii* trophozoites occurred within 24 hr in both scarred and unscarred corneas. The amoebae did not appear to be particularly associated with the scars but had migrated throughout the stroma. The role of Bowman's layer in inhibiting penetration to the corneal stroma therefore remains uncertain.

The definitive method adopted, which also demonstrated invasion by V-EYE and H-EYE, merely involved incubating human cornea epithelial side down on a contact lens supported in a contact lens holder. As far as I am aware this is the first reported study of the ability of *Vahlkampfia* or *Hartmannella* to invade human tissue *in vitro* in organ culture.

It should be noted, however, that the human corneas used in this study were eyebank rejects which had been stored in tissue culture medium for 1-2 months and hence possessed little or no epithelium (personal observations). In contrast the rabbit eyes were received fresh and so had a healthy epithelium. It is therefore likely that the lack of epithelium on the human cornea facilitated demonstration of stromal invasion (see Sections 4.4.2.3 & 4.4.3.2 concerning the relative insusceptibility of epithelial cells to the cytopathic effects of amoebae).

4.4.1.2 Post-inoculation studies

Eyebank corneas, which had been stored in tissue culture medium for 1-2 months, were also used for inoculation studies. As already mentioned (Section 2.1.2.2) keratocyte depletion occurs with time, although much less rapidly than loss of epithelial cells. This may partly account for the slightly different time course observed for progression to encystment with different inoculations. The other factor is, of course, the inoculating dose of amoebae. Following a steep learning curve, with practice, the rather fiddly technique became more consistently reliable, but the number

of amoebae successfully inoculated could not be controlled with any degree of accuracy. As described in Section 2.3.1.2, both the inoculation method and fixation had been optimised to enhance morphological preservation in this *in vitro* model of stromal amoebal keratitis.

Interestingly it was observed that relatively little disruption of tissue architecture occurred, even with marked migration of the trophozoites throughout the tissue and depletion of keratocytes. This is in contrast to human pathological specimens, described in detail in Section 1.3.2, which demonstrate destruction of lamellar collagen and reactionary fibrosis (Blackman *et al.*, 1984; Garner, 1988 & 1993); these studies suggest that much of the damage observed *in vivo* is actually due to the efforts of the immune system to eradicate the infectious agent. As described in Section 1.3.3, studies on various animal models (rat, rabbit, pig, Chinese hamster) also suggest that the immune response evoked is associated with tissue disruption (Badenoch *et al.*, 1990; Cote *et al.*, 1991, He *et al.*, 1992; van Klink *et al.*, 1993 respectively). This was investigated in detail in a rat model by Larkin & Easty (1990 & 1991).

Use of a renal biopsy needle to take a core biopsy from such paraffin-embedded blocks of tissue enabled serial sections to be cut all the way through a tissue without destroying the whole of the original tissue. It should, however, be noted that such core biopsies only demonstrate a selected region of the original tissue. While this method might usefully be employed to obtain a sample of a precious pathological specimen, the area of the core biopsy would require to be selected with care in order to include diseased tissue while leaving some of the same for future study.

4.4.2 Cytopathogenicity for Corneal Cells in Tissue Culture

4.4.2.1 Time/concentration study of the cytopathic effect of Acanthamoeba *on keratocytes*

Co-incubation of a 10^6/ml concentration of *Acanthamoeba castellanii* with keratocytes led to progressive destruction of the majority of the 'monolayer' within 24 hr. Destruction of the remaining cells occurred at a lesser rate. This may at least be partly due to the fact that the cells that survive longer tend to be relatively protected (i.e. physically more difficult to attack because of clumping). Interestingly the reaction did not proceed to completion.

This is even more noticeable in the case of lesser concentrations of *Acanthamoeba castellanii*, where rounding-up/encystment occurred in the face of a plentiful food source. The explanation is unknown but may be related to a build up of waste products in the environment. Certainly the acidity of the culture medium was observed to increase with time (i.e.. the pH indicator changed from red to yellow). Similar rounding-up/encystment in spite of a plentiful food source is also observed when culturing amoebae on heat killed bacteria. In contrast, Larkin *et al.* (1991) reported that, with a concentration of amoebae sufficient to produce a cytopathic effect, then amoebae encysted only following near complete destruction of the cells. They also described an interesting experiment showing that excystment of *Acanthamoeba polyphaga* occurred on confluent rabbit cells but not on non-confluent cells for trophozoite concentrations of 5×10^2 and 5×10^4/well in a 24 well plate.

The results obtained for 10^4/ml *Acanthamoeba castellanii* on keratocytes illustrate that an initial small amount of damage was reversible. Reversal of the damage could occur by increase in cell numbers or increase in cell area. The latter could be achieved by cell respreading (see CD-ROM). The logic behind switching the medium from EGM 20% to EGM 2% for the course of the experiment was to minimise further cell growth. Cells maintained in a nutritionally-poorer medium grow more slowly than cells maintained in a

comparatively rich medium: if cells are switched from the latter to the former they also undergo a period of retarded growth as they adapt (personal observations). The effect of this is, however, by no means absolute and cell growth was not completely inhibited by the treatment described above. With 10^4/ml *Acanthamoeba castellanii*, the dynamics of the co-incubation were such that the rate of increase in cell numbers or cell area was sufficient to reverse the small amount of initial damage. This was only detected, however, because at this concentration the dynamics of the interaction were such that the amoebae were unable 'to get the upper hand'.

Concentrations of *Acanthamoeba castellanii* below 10^4/ml failed to demonstrate any observable cytopathic effect, in spite of the fact that trophozoites were migrating over the surface of the cells and did not round-up/encyst until approximately day 4-5. This agrees with the results of Larkin *et al.* (1991) that also describe a 'cut-off' point. Presumably some cell damage was occurring but this was effectively masked by cell growth, or possibly cell respreading (see CD-ROM). Obviously this has implications in human disease concerning inoculation dose and the ability to establish on-going stromal keratitis (Section 1.3.4).

4.4.2.2 Comparison of the cytopathic effects of Acanthamoeba, Vahlkampfia *and* Hartmannella *on keratocytes*

To my knowledge there are no reported similar studies of the cytopathic effects produced by *Vahlkampfia* or *Hartmannella*. The comparison of the relative cytopathogenicity of representatives of the 3 genera of amoebae indicate that, although the representatives of the 3 genera of amoebae at a concentration of 10^6/ml can all produce near destruction of the monolayer within 24 hr, the initial rate of cell destruction is greater with the axenically grown *Acanthamoeba* AC_a than with the monoxenically cultivated V-EYE and H-EYE. In interpretation of this result it should be borne in mind that axenisation has been shown to decrease virulence in *Acanthamoeba* (Stevens & O' Dell, 1974, Cursons & Brown, 1978). This would suggest that the true difference in

cytopathogenicity has been masked and is in fact greater than suggested. The choice of axenic *Acanthamoeba* was, however, based on preliminary experiments which had demonstrated that both AC_a and AC_b were capable of near destruction of the monolayer within 24 hr (i.e. no difference).

It may also be relevant that reassociation with bacteria has been found to increase virulence in *Entamoeba histolytica* (Wittner & Rosenbaum, 1970; Bracha & Mirelman, 1984). That this may also occur with *Acanthamoeba* is perhaps suggested by the requirement for *Corynebacterium* for successful establishment of keratitis in the rat model described by Badenoch (Badenoch *et al.*, 1990). If this is the case then, even if there was no difference in cytopathogenicity in this system between axenic and monoxenic *Acanthamoeba*, the use of bacteria-fed *Vahlkampfia* and *Hartmannella* may feasibly have skewed the results in their favour.

It should also be noted, however, that the observed difference in initial cytopathogenicity may in fact be simply artefact, due to technical factors. The reason AC_a was chosen to conduct the experiment was that axenic cultures are much easier to cultivate consistently. Although all trophozoites used in the experiments were in the log phase of growth, the axenic cultures were growing much more rapidly. This was particularly true with regard to V-EYE, which prior to this experiment had been growing particularly poorly. It may therefore have been this factor that led to the initial more rapid period of cell destruction by *Acanthamoeba*.

Finally, the results should also be interpreted with the proviso that any comparison between protozoal species is complicated because, not only do changes in culture conditions affect amoebal cytopathogenicity (Section 1.3.5), but the conditions for maximal cytopathogenicity may be inherently different (Section 1.3.4). Furthermore, even if the results for the ability to produce a cytopathic effect on cultured cells are directly comparable, they are not

absolute proof that all 3 genera are capable of causing infection of the human cornea. The relationship between the ability to produce a cytopathic effect *in vitro* and their ability to produce disease *in vivo* is not clear (Section 1.3.5).

4.4.2.3 Comparison of the susceptibility of epithelial cells and keratocytes to **Acanthamoeba**

There are no similar studies of the relative susceptibility of human epithelial cells and keratocytes to *Acanthamoeba* in the literature. Brown (1978) had, however, tentatively suggested that mouse embryo epithelial cells appeared to be relatively resistant to destruction by *Naegleria fowleri.* At least in the model system described in this study, keratocytes were manifestly more susceptible than epithelial cells to the cytopathic effect produced by *Acanthamoeba castellanii* at the different concentrations tested. The vulnerability of keratocytes compared to epithelial cells was most marked early in the co-incubation period. This result was verified for *Acanthamoeba castellanii* at a concentration of 10^6/ml not only by the more detailed quantification studies but also by the time lapse video analysis. Analysis of the former results, however, reveals a distinct 'hump' at the 6 hr position on the x-axis of the graph of destruction of keratocytes (Fig 4.3). This is also observed, but to a very much lesser extent, for epithelial cells. There are several possible explanations for this.

First, the 'hump' may simply be an artefact. It is possible that the coverslips representing 3 and 6 hr of incubation may have been switched in error at some point during processing, This is considered unlikely: it is observed for both types of cells and also the results obtained by image analysis concur with the visual impression of the ongoing process An alternative explanation is that the cells were not equally distributed between the wells at the commencement of the experiment (i.e. if there were comparatively fewer cells in the wells fixed at 3 hr or comparatively greater numbers in the wells fixed at 6 hr). This is also considered unlikely due to the method of aliquoting. When the keratocytes were plated out the night before, they were prepared as a stock suspension of 50 000/ml which was then pipetted, row by row, into the individual wells.

Although fixation of the cells at the various time intervals was also carried out row by row, this is unlikely to be the cause of the observed blip because the cells were aliquoted with a 10 ml pipette (i.e. 10 wells at a time). Also, the same blip is observed for epithelial cells, which were aliquoted 7-10 days before and then allowed to grow to confluence.

Second, the 'hump' may represent a real phenomenon. In that case, as the values were compared to the same 24 hr control in each case, there is actually a greater area of cells present at 6 hr than at 3 hr. This would only be possible if the cells had multiplied in number or grown in area (i.e. respread). As described above (Section 4.4.2.1) growth of cells is retarded by conducting the experiment in EGM 2% rather than EGM 20%, but is not completely inhibited. Theoretically, growth/respreading of cells could account for the observed blip; the dynamics of the ongoing process would have to be such that, after an initial period of cell destruction, cell growth/respreading briefly exceeded cell loss, only to be overtaken once more by the rate of cell destruction. The rate of cell growth is unlikely to vary much during the course of the experiment. The rate of destruction of cells clearly does, however, at least partially due to the relative ease of destruction of cells.

Whatever the explanation for the observed 'hump', it does not alter the finding that increased susceptibility of keratocytes compared to epithelial cells to the cytopathic effect produced by *Acanthamoeba castellanii* can be largely accounted for by an increased rate of destruction in the initial period, involving at least the first 3 hr of co-incubation.

4.4.3 Mechanism of Cytopathogenicity

4.4.3.1 Production of cytopathic effects in the presence of physical contact

Studies by time lapse video and SEM emphasise the importance of physical factors in the production of a cytopathic effect both with regard to detachment

of cells from substrate and subsequent trogocytosis of partially disrupted cells. These results largely agree with the process of cell damage described for *Naegleria fowleri* by Brown (1978) who also demonstrated the importance of functioning pseudopodia (Brown, 1979). Amoebostomes are sucker-like structures that can engulf particles that are known to be involved in phagocytosis by *Naegleria* (Marciano-Cabral & John, 1983). They have also been described for *Acanthamoeba* (Diaz *et al.*, 1991; Pettit *et al.*, 1996), but were not observed in the present study. The latter paper does, however, state that their demonstration required freshly prepared 2% buffered glutaraldehyde at 37 ^{0}C. This fixation was not performed in the current study, but nor is it mentioned as necessary by Diaz *et al.* (1991).

It has been reported that amoebae selectively phagocytose nucleated erythrocytes and indeed enucleate them (Chi *et al.*, 1959). In the present experiments trophozoites were certainly observed apparently entering, and even encysting within, partially disrupted cells (Fig 4.7). Tyndall *et al.* (1979) also describe *Acanthamoeba* inserting pseudopodia and apparently entering cultured tumour cells. Definitive proof of this, however, would require serial sectioning with reconstruction: the appearance may in fact be artefactual even in sectioned specimens, due to plane of section [see explanation in Brown (1979) where he describes apparent intracellular location of *Naegleria fowleri* by transmission electron microscopy].

4.4.3.2 Production of cytopathic products

Although mechanical factors appeared to play an important role in the production of the observed cytopathic effect, complete destruction of the cells was also achieved in the absence of physical contact. The latter process, however, required a longer exposure. This would suggest that mechanical and secretory factors normally act in concert. It was less marked with medium conditioned with the intracellular contents of lysed amoebae than when amoebae were incubated in the transwell apparatus. Furthermore, the cytopathic agent(s) must have been constituitively produced as the amoebae

from which the cell-free parasite lysates were derived were axenically cultured.

The transwell results are particularly interesting because the amoebae managed to initiate and sustain cell damage without being in direct contact with the cells. Presumably the amoebae did not re-encyst in appreciable numbers during this time (c.f. to concentrations of 10^5/ml/well or less in direct contact with keratocytes). That conditioned medium failed to produce a cytopathic effect in the absence of live amoebae suggests that the factors produced may be short-lived. Other authors have demonstrated specific enzyme activity by amoeba-conditioned medium (He *et al.*, 1990; Mitro *et al.*, 1994) although most studies concerned amoeba lysates (Section 1.3.5). Biochemical analysis of cytopathic products was, however, outwith the scope of this project (see Section 5.2.2).

The significance of the finger-like projections illustrated in Figure 4.6B is unknown. Since these experiments were performed, Pettit *et al.* (1996) have also reported the presence of similar projections, which they term digipodia, in several different *Acanthamoeba* sp exposed to neuroblastoma cells. These authors suggest that the digipodia may be in the process of ingesting a filamentous thread-like extension from the target cells, or, alternatively, secreting material into the target cells. The former appearance was not observed in the current study; it may indeed be artefact, secondary to shrinkage during processing for electron microscopy.

Pettit *et al.* (1996) also suggest that the digipodia may be associated with apoptosis of the target cell. They state that effector cell-target cell contact was associated with margination of chromatin and membrane blebbing which are characteristic morphological changes observed in cells undergoing apoptosis. The occurrence of apoptosis in a subject of the target cell population was demonstrated by the TUNEL (Tdt-mediated dUTP nick end labelling) technique which detects enzymatically-labelled DNA fragments/strand breaks. In my experience, however, digipodia were not associated with cell blebbing,

nor, indeed was blebbing observed in the general cell population, whether the amoebae were in direct contact with the cells, or separated by a membrane in the transwell apparatus. Furthermore, apoptosis was not involved in the disintegration of cells exposed to secretory products only, as assessed using Oncor APOPTAG™ ISH Apoptosis Detection Kit. As this procedure was only performed on cells from the transwell apparatus, it is possible that effector cell-target cell contact is a prerequisite. Alizadeh *et al.* (1994), however, have demonstrated that *Acanthamoeba castellanii* extract alone produced cell death by the process of apoptosis. Their claim was based on the characteristic morphological changes as assessed by light microscopy and SEM, characteristic DNA fragmentation patterns on electrophoresis and the results of propidium iodide staining and flow cytometry. Although the paper describes apoptosis on tumour cells exposed to *Acanthamoeba castellannii* they also state that the same is true of pig corneal epithelial cells. The results, however, were not presented.

Investigation of the actual mechanism of production of cell damage also suggests an explanation for why keratocytes may be more susceptible than epithelial cells, especially in the initial phases of co-incubation (Section 4.2.2.3 & Fig 4.3). Keratocytes are typical fibroblastic type cells which do not form a true monolayer (Fig 4.4B). Epithelial cells in contrast do form a monolayer with individual cells connected by tight junctions with desmosomes (Fig 4.4A). The results suggested that the initial stage of cell damage appeared to involve mechanical infiltration of the amoebae between cells. It seems reasonable to suggest that this would be much more difficult with epithelial cells than with keratocytes (See CD-Rom). Once the attachments to the substratum and other cells had been loosened, however, with the appearance of retraction fibres (Fig 4.4C-H) the epithelial cells would be no more protected from attack than keratocytes. The pattern of cell damage suggested by this scenario would concur with that illustrated in Fig 4.3.

5. CONCLUSIONS

5.1 Diagnostic Methodologies

5.1.1 Histology and Immunodiagnosis

Although calcofluor white failed to label trophozoites effectively, it has also been shown to be a vital stain, differentiating living and dead cells by staining dead cell nuclei (Fischer *et al.*, 1985). Theoretically this could allow monitoring of the effectiveness of on-going therapy in *in vitro* models and even in clinical specimens, following proper evaluation. Failure to effectively demonstrate trophozoites may not particularly limit its potential in this role.

Successful demonstration of trophozoites in pathological specimens by non-specific stains is still required. It awaits the identification and exploitation of some biochemical difference/difference in staining properties between these cells and those from which they require to be distinguished (in particular keratocytes and macrophages). For example, a combined stain for esterase or alkaline phosphatase in combination with methyl green has been described (Tyndall *et al.*, 1979). This differentiates mammalian cells (green nucleus and tan cytoplasm) from amoeboid cells (dark brown or black with the esterase or alkaline phosphatase stains respectively). This, however, is unsuitable for diagnostic application to pathological specimens because enzyme activity is destroyed by fixation.

A pan-amoeba antiserum is obviously useful in cases of suspected amoebal keratitis, but it is also desirable to be able to identify the genus responsible because alternative therapies may be more effective in keratitis associated with *Vahlkampfia* or *Hartmannella*. In contrast to polyclonal antisera, monoclonal antibodies recognise a single antigen and are therefore more specific. At least theoretically, different monoclonals capable of recognising and differentiating the three different genera could be manufactured, as has been achieved for *Naegleria* and *Acanthamoeba* (Flores *et al.*, 1990). This would be dependent

on the existence of antigenic differences, as has been demonstrated for *Hartmannella* and *Acanthamoeba* (Visvesvara & Balamuth, 1975). It would, however, be potentially time consuming with no guarantee of success; it is essentially a hit and miss approach.

5.1.2 Molecular Biology Techniques

As already stated, for diagnosis of such a ubiquitous organism, the extreme sensitivity of PCR may be a weakness rather than a strength. Gast δ Byers (1995), however, describe much reduced sensitivity for PCR amplification of amoebal DNA in the presence of both human cell and bacterial DNA. The influence of potentially co-contaminated formalin-fixed corneal tissue on the sensitivity, and thus the suitability, of PCR for diagnosis of *Acanthamoeba* keratitis should be assessed for P1/P2 and other potentially useful primers. It is also worthy of note that archival studies are theoretically possible with PCR, both on DNA extracted from formalin-fixed paraffin-embedded tissue blocks (Impraim *et al.*, 1987) and also directly on sections (Shibata *et al.*, 1988).

Even if PCR does not prove useful in the routine diagnostic laboratory, it may be productive for typing or sequencing for unusual isolates. Its potential in this role has already been demonstrated by Ledee *et al.* (1996). They describe not only identification of a species not previously associated with keratitis, but also unequivocal confirmation of the epidemiological association between a keratitis-causing strain of *Acanthamoeba*, the contact lens storage case and the domestic water supply. It should be emphasised, however, that the significance of detailed molecular biological analyses requires cautious interpretation, even by molecular biologists specialising in amoebal genetics, due to the rapid expansion of data in this field and the resulting confusion concerning its phylogenetic implications (see Section 1.1.2).

With regard to PCR identification of amoebae other than *Acanthamoeba*

castellanii, the significance of the faint ghost bands at the 122-bp position and also the non-specific bands, is unknown. Reproducibility might theoretically be improved by further optimisation and standardisation of the PCR procedure. [See Kerr (1994) for a summary of uses and review of pitfalls associated with various PCR-based typing techniques]. Cloning and sequencing of the 122-bp ghost band in each case would reveal whether this was identical to the sequence expected with *Acanthamoeba castellanii* (and therefore potentially due to contamination) or whether there were indeed base pair differences present (suggesting that the target genome contains slight mismatches producing the resulting fainter amplification product). Restriction digestion of the amplified product may also be able to resolve differences at the primary sequence level as described in Vodkin *et al.* (1992). If amplification of the *Vahlkampfia* and *Hartmannella* genomes is, in fact, occurring, then sequencing of the resulting products would yield information potentially useful to the design of specific primers/probes.

ISH is not as prone to false positive results as PCR, but the labelling achieved in this study was patchy. Further modification of the protocol with regard to poor penetration is therefore required. At present, the method presents no advantages over immunodiagnosis and indeed failed to label V-EYE. These preliminary data do, however, demonstrate the feasibility of identifying protozoa in tissue by ISH. Furthermore, unlike antibody production, with the aid of genebank data, specific probes to differentiate the 3 genera should be relatively easy to design and manufacture. Programs which allow comparison of intended sequences with all other known DNA/RNA sequences are available to facilitate assessment of likely specificity. Relevant sequencing data for *Vahlkampfia* and *Hartmannella* genomes, however, are not yet available for analysis.

5.2 Pathogenicity of Acanthamoeba, Vahlkampfia and Hartmannella

5.2.1 Pathogenicity for Corneal Tissue in Organ Culture

Although the *in vitro* models described herein have yielded useful information, they are associated with major drawbacks. For example epithelial cells in culture form only a monolayer while keratocytes are not surrounded by stromal elements and eyebank corneas have little, if any, residual epithelium. Investigation of the ability of V-EYE and H-EYE to produce keratitis in an animal model would therefore be strong evidence that they are indeed pathogenic to humans. The resulting pathology could also be compared to that produced by *Acanthamoeba,* with consideration of any implications concerning pathogenesis and prognosis of keratitis associated with the 3 different genera. Various animal models have been described (Section 1.3.3) but all required considerable facilitation to establish on-going keratitis. A more 'physiological' *in vivo* model of *Acanthamoeba* keratitis is therefore awaited.

In the *in vitro* models described in this study there is no involvement of the immune system. As there was little tissue disruption in either inoculated or invaded corneas, this suggests that much of the pathology observed in clinical pathology specimens is due to the efforts of the immune system to resolve the infection. This could be further investigated by incubating or inoculating corneal tissue with *Acanthamoeba* and immune cells primed against *Acanthamoeba*.

In the experiments described herein there was also no attempt to treat the infection *in vitro*. Further experiments, utilising human corneal specimens exposed to amoebae with or without therapy, and comparing the pathology produced *in vitro* with that of clinical pathological specimens, should help to confirm the contribution of the anti-amoebal therapy to the observed tissue disruption.

The optimum therapy for keratitis associated with *Vahlkampfia* or *Hartmannella* may be different from that for *Acanthamoeba* keratitis. Indeed, it has been demonstrated *in vitro* that *Vahlkampfia* and *Hartmannella* are more sensitive to Amphotericin B, but toxicity limited usage of this compound in Patient GM (Aitken *et al.*, 1996). The corneal models described in this study could also be used to assess the efficacy and toxicity of potential anti-amoebal chemotherapeutic agents, as indeed could co-cultures of amoebae and corneal cells.

Studies with fresh human corneas would also demonstrate whether a healthy epithelium constitutes an effective barrier to amoebal invasion. Further refinement of the model system to demonstrate invasion would be required. At present the cornea is incubated epithelium-side down on a contact lens, being gently introduced after the amoebae have settled out of suspension. The amoebae, however, are able to migrate and could theoretically invade through the cut edge of the sclera, or indeed through the endothelium, though they would then have to also breach Descemet's membrane. These problems need to be addressed. For example, tissue glue could be used to seal the edges of the cornea.

Radial keratoneuritis, an unusual clinical sign with linear infiltrates apparently situated along the corneal nerves, is virtually pathognomonic for *Acanthamoeba* keratitis (Moore *et al.*, 1986). Whether the infiltrates are due to inflammatory cells or the amoebae themselves is uncertain (Section 1.3.4). This could be investigated on clinical specimens by light or transmission electron microscopy, although the process would be laborious.

Experimentally an affinity for the optic nerve has been suggested by studies involving intra-ocular inoculation of *Acanthamoeba culbertsoni* (Schaegel & Culbertson, 1972). Inflammation of other ocular tissues secondary to

Acanthamoeba keratitis has been described (Key *et al.*, 1980; Mannis *et al.*, 1986; Johns *et al.*, 1988; Lindquist *et al.*, 1990; Burke *et al.*, 1992). There are, however, only 4 cases of documented *Acanthamoeba* infection outwith the cornea (Jones *et al*, 1975; McLellan & Coster, 1987; Dougherty *et al.*, 1994; Heffler *et al.*, 1996), and the first and last of these were associated with haematogenous spread (Section 1.3.4). The ability of amoebae to invade non-corneal ocular tissues could be further investigated either *in vitro* on whole human eyes or *in vivo* on a suitable animal model. It would be of particular interest with regard to V-EYE and H-EYE in view of the current debate surrounding the potential of *Vahlkampfia*, and more particularly *Hartmannella*, to be pathogenic (Section 1.1.1).

5.2.2 Pathogenicity for Corneal Cells

In view of the proposed propensity of the 3 genera of free-living amoebae for infecting neural tissue (Section 1.3.4), it would be of interest to assess the cytopathic effect produced on neural cells compared to other corneal cells.

The role of co-infection with bacteria in establishment of ongoing corneal infection has not been fully elucidated (see Section 1.3.4). While not a prerequisite, co-infection may, theoretically at least, facilitate establishment of ongoing infection in humans. This interaction could be investigated in the corneal model or in the corneal cell model.

V-EYE would be of particular interest in this regard as repeated attempts at axenisation were thwarted by the recurrent growth of bacteria after approximately a week post-acid wash. This suggested that the bacteria were harboured intracellularly; naturally occurring endosymbionts have certainly been previously described (Fritsche *et al.*, 1993; Yagita *et al.*, 1995). Indeed the interaction between free-living amoebae and intracellular bacteria, first recognised by Rowbotham (1980), is the subject of much topical interest (Allen *et al.*, 1998; Boman & Berk, 1998; Cirillo *et al.*, 1998; Visvesvara & Leitch,

1998). Inhibition of amoebal growth by the presence of particular bacteria has been described (Qureshi *et al.*, 1993). Although in our experience the presence of intracellular bacteria apparently hampered growth of the *Vahlkampfia*, they could also theoretically have contributed to the ability to invade and produce stromal infection.

Investigation of the putative barrier-function of the tight junctions of the epithelial monolayer could also be performed on the cell culture model. Epithelial tight junctions may be disrupted by specific anti-E Cadherin antibodies (Yoshida-Noro *et al.*, 1984, Shimoyama *et al.*, 1989) or by hepatocyte scatter factor (Gherardi *et al.*, 1989). Comparison of the susceptibility to cell damage of an intact monolayer compared to a disrupted monolayer would allow assessment of the protective effect of the normal growth pattern of epithelial cells. Alternatively, epithelial cells may be relatively protected due to some other factor such as mucous secretion from the conjunctiva. This could also be investigated, although always with the proviso that cultured cells do not accurately represent the situation *in vivo*.

5.2.2 Mechanism of Cytopathic Effect

Further studies are required concerning the virulence of all 3 genera of amoebae, both as regards the contribution of physical attack and the role of secretions. Physical consumption of the cells could be confirmed by marker uptake studies, as demonstrated for *Naegleria* (Brown, 1979). A role for trogocytosis, as opposed to phagocytosis, could be confirmed by release of a membrane-bound marker into the medium as described for ^{51}Cr and *Naegleria* (Fulford *et al.*, 1985). A correlation between virulence and certain secretions has been suggested for *Acanthamoeba* (Section 1.3.5). The secretions of *Vahlkampfia* and *Hartmannella* have not yet been analysed in any detail. The presence of amoebostomes, not demonstrated on any of the 3 genera in this study, could be further investigated using warmed fixative as described for *Naegleria* (Marciano-Cabral & John, 1983). The finger-like projections

observed for *Acanthamoeba castellanii* merit further investigation both with regard to their role and whether they occur with *Vahlkampfia* and *Hartmannella*. The potential involvement of apoptosis is also still unresolved. Only cells exposed to *Acanthamoeba castellanii* in the absence of physical contact were tested by the Oncor APOPTAG ™ ISH Apoptosis Detection Kit in this study. Cells damaged in the presence of physical contact could also be analysed and so too could cells exposed to *Vahlkampfia* and *Hartmannella*. Further investigations could include scanning and transmission electron microscopy for demonstration of characteristic cell blebbing and nuclear condensation respectively and gel electrophoresis for detection of a characteristic banded pattern of DNA (Alizadeh *et al.*, 1994).

The *Vahlkampfia* and *Hartmannella* species isolated from patient GM's contact lens case (Aitken *et al.*, 1996) are also available for comparison with V-EYE and H-EYE as regards both cytopathogenicity and actual secretions. This would allow investigation of whether adaptation to growth in the intraocular environment had occurred, perhaps rendering a primarily non-infective organism virulent.

Prior to further studies using *Vahlkampfia* and *Hartmannella* sp, axenisation is a priority. Although difficult to achieve, once established this greatly facilitates growth and harvesting of large numbers of amoebae, replicating rapidly, in the log phase of growth. Unsuitable conditions leads to marked stunting, not just of growth rate but also in size (personal observations). It is thought that this may have contributed to the observed differences between the different genera, in particular with regard to V-EYE. The growth requirements of these rather fastidious organisms are, however, poorly understood. Much trial and error is therefore required with this rather laborious and painstaking step-by-step procedure. Greater understanding of the nutritional and environmental requirements of these organisms may also reveal the cause of encystation in the face of a plentiful food supply. This is of particular interest with respect to the cut-off effect demonstrated with lower numbers of amoebae on cultured cells;

such factors may have some influence over whether or not an ongoing stromal infection is produced *in vivo*.

Apart from facilitating the experimental procedure, axenisation of all species would theoretically allow more direct comparison of the cytopathic effects produced. Axenic growth is, however, known to decrease virulence with time (Stevens & O'Dell, 1974, Cursons & Brown, 1978); this can be re-acquired by animal (Stevens & O'Dell, 1974) or cell passage (John & John, 1994). The different genera would also most probably require different axenic media for optimal growth, further confounding the comparisons. In fact, it has been shown that varying culture conditions can have profound effects (Section 1.3.5) and thus may actually mask potential differences between the ocular and contact lens isolates. These effects would, of course, have to be taken into account in interpretation of the ability of any particular species to produce a cytopathic effect.

BIBLIOGRAPHY

Aitken, D., Hay, J., Kinnear, F.B., Kirkness, C.M., Lee, W.R. & Seal, D.V. (1996) Amebic keratitis in a wearer of disposable contact lenses due to a mixed *Vahlkampfia* and *Hartmannella* infection. *Ophthalmology* **103,** 485-494.

Alexandrakis, G., Miller, D. & Huang, A.J.W. (1998) Amebic keratitis due to *Vahlkampfia* infection following corneal trauma. *Archives of Ophthalmology* **116,** 950-951.

Alizadeh, H., Pidherney, M.S., McCulley, J.P. & Niederkorn, J.Y. (1994) Apoptosis as a mechanism of cytolysis of tumor cells by a pathogenic free-living amoeba. *Infection and Immunity* **62,** 1298-1303.

Allen, S.D., Emery, C.L., Siders, J.A. & Harris, E.E. (1998) Survey of interactions between species of *Acanthamoeba* and bacteria involved in respiratory tract infections. *Eighth International Conference on the Biology and Pathogenicity of Free-living Amoebae* **(Abstracts).**

Altshul, S.P., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) Basic local alignment research tool. *Journal of Molecular Biology* **215,** 403-410

Auran, J.D., Starr, M.B. & Jakobiec, F.A. (1987) *Acanthamoeba* keratitis: A review of the literature. *Cornea* **6,** 2-26.

Bacon, A.S., Dart, J.K.G., Ficker, L.A., Matheson, M.M. & Wright, P. (1993a) *Acanthamoeba* keratitis. The value of early diagnosis. *Ophthalmology* **100,** 1238-1243.

Bacon, A.S., Frazer, D.G., Dart, J.K.G., Matheson, M., Ficker, L.A. & Wright, P. (1993b) A review of 72 consecutive cases of *Acanthamoeba* keratitis, 1884-1992. *Eye* **7,** 719-725.

Badenoch, P.R., Adams, M. & Coster, D.J. (1995) Corneal virulence, cytopathic effect on human keratocytes and genetic characterization of *Acanthamoeba*. *International Journal for Parasitology* **25,** 229-239.

Badenoch, P.R., Johnson, A.M., Christy, P.E. & Coster, D.J. (1990) Pathogenicity of *Acanthamoeba* and a Corynebacterium in the rat cornea.

Archives of Ophthalmology **108,** 107-112.

Bancroft, J.D. & Stevens, A. (1996) *Theory and practice of histological techniques,* 4th Ed. New York: Churchill Livingstone.

Baverstock, P.R., Illana, S., Christy, P.E., Robinson, B.S. & Johnson, A.M. (1989) srRNA evolution and phylogenetic relationships of the genus *Naegleria* (Protista:Rhizopoda). *Molecular Biology and Evolution* **6,** 243-257.

Beattie, A.M., Slomovic, A.R., Rootman, D.S. & Hunter, W.S. (1990) *Acanthamoeba* keratitis with two species of *Acanthamoeba. Canadian Journal of Ophthalmology* **25,** 260-262.

Blackman, H.J., Rao, N.A., Lemp, M.A. & Visvesvara, G.S. (1984) *Acanthamoeba* keratitis successfully treated with penetrating keratoplasty: suggested immunogenic mechanisms of action. *Cornea* **3,** 125-130.

Bogler, S.A., Zarley, C.D., Burianek, L.L., Fuerst, P.A. & Byers, T.J. (1983) Interstrain mitochondrial DNA polymorphism detected in *Acanthamoeba* by restriction endonuclease analysis. *Molecular and Biochemical Parasitology* **8,** 145-163.

Boman, W.C. II & Berk, S.G. (1998) Production of vesicles containing live *Mycobacterium avium* by *Acanthamoeba polyphaga. Eighth International Conference on the Biology and Pathogenicity of Free-living Amoebae* **(Abstracts).**

Borochovitz, D., Martinez, A.J. & Patterson, G.T. (1981) Osteomyelitis of a bone graft of the mandible with *Acanthamoeba castellanii* infection. *Human Pathology* **12,** 573-576.

Bottone, E.J., Madayag, R.M. & Qureshi, M.N. (1992a) *Acanthamoeba* keratitis -synergy between amebic and bacterial cocontaminants in contact lens care systems as a prelude to infection. *Journal of Clinical Microbiology* **30,** 2447-2450.

Bottone, E.J., Qureshi, M.N. & Asbell, P.A. (1992b) A simplified method for demonstration and isolation of *Acanthamoeba* organisms from corneal scrapings and lens care systems. *American Journal of Ophthalmology* **113,**

214-215.

Bovee, E.C. (1985) Class Lobosea Carpenter, 1861 in: *An illustrated guide to protozoa,* eds. Lee, J.J., Hutner, S.H. & Bovee, E.C. pp158-211. Lawrence, Kansas: Society of Protozoologists.

Bowers, B. & Korn, E.D. (1968) The fine structure of *Acanthamoeba castellanii.* I. The trophozoite. *The Journal of Cell Biology* **39,** 95-111.

Bowers, B. & Korn, E.D. (1969) The fine structure of *Acanthamoeba castellanii* (Neff strain). II. Encystment. *The Journal of Cell Biology* **41,** 786-805.

Bracha, R. & Mirelman, D. (1984) Virulence of *Entamoeba histolytica* trophozoites. Effects of bacteria, microaerobic conditions and metronidazole. *Journal of Experimental Medicine* **160,** 353-368.

Brooks, J.G. Jr., Coster, D.J. & Badenoch, P.R. (1994) *Acanthamoeba keratitis*: resolution after debridement. *Cornea* **13,** 186-189.

Brown, S. & De Jonckheere, J.F. (1994) Identification and phylogenetic relationships of *Vahlkampfia* spp. (free-living amoebae) by riboprinting. *FEMS Microbiology Letters* **115,** 241-246.

Brown, T. (1978) Observations by light microscopy on the cytopathogenicity of *Naegleria fowleri* in mouse embryo-cell cultures. *Journal of Medical Microbiology* **11,** 249-259.

Brown, T. (1979) Observations by immunofluorescence microscopy and electron microscopy on the cytopathogenicity of *Naegleria fowleri* in mouse embryo-cell cultures. *Journal of Medical Microbiology* **12,** 363-371.

Burke, J.P., Webber, S.K., Kerr-Muir, M.G., Talbot, J.F. & Parsons, M.A. (1992) *Acanthamoeba polyphaga* panophthalmitis. *Cornea* **11,** 274-275.

Byers, T.J., Hugo, E.R. & Stewart, V.J. (1990) Genes of *Acanthamoeba*: DNA, RNA and protein sequences (a review). *Journal of Protozoology* **37,** 17S-25S.

Byers, T.J., Kim, B.G., King, L.E. & Hugo, E.R. (1991) Molecular aspects pf the cell cycle and encystment of *Acanthamoeba*. *Reviews of Infectious*

Diseases **13 (Suppl 5):** S378-S384.

Casella, J.P., Crosier, S. & Hay, J. (1989) Needle-biopsy of paraffin blocks to obtain tissue for electron microscopy. *Medical Laboratory Science* **46,** 378.

Castellani, A. (1930) Amoeba found in a culture of yeast: preliminary note. *The Journal of Tropical Medicine and Hygiene* **33,** 160.

Centeno, M., Rivera, R., Cerva, L., Tsutsumi, V., Gallegos, E., Calderon, A., Ortiz, R., Bonilla, P., Ramirez E. & Suarez, G. (1996) *Hartmannella vermiformis* isolated from the cerebrospinal fluid of a young male patient with meningoencephalitis and bronchopneumonia. *Archives of Medical Research* **27,** 579-586.

Chew, S-J., Beuerman, R.W., Assouline, M., Kaufman, H.E., Barron, B.A. & Hill, J.M. (1992) Early diagnosis of infectious keratitis with in vivo real time confocal microscopy. *Contact Lens Association of Ophthalmologists Journal* **18,** 197-201.

Chi, L., Vogel, J.E. & Shelokov, A. (1959) Selective phagocytosis of nucleated erythrocytes by cytotoxic amebae in cell culture. *Science* **25 December,** 1763.

Cirillo, J.D., Falkow, S. & Tompkins, L.S. (1994) Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infection and Immunity* **62,** 3254-3261.

Clark, B.J., Harkins, L.S., Munro, F.A. & Devonshire, P. (1994) Microbial contamination of cases used for storing contact lenses. *Journal of Infection* **28,** 293-304.

Clark, C.G. & Cross, G.A.M. (1988) Small-subunit ribosomal RNA sequence from *Naegleria gruberi* supports the polyphyletic origin of amoebas. *Molecular Biology and Evolution* **5,** 512-518.

Connor, R., Hay, J., Mead, A.J.C. & Seal, D.V. (1993) Reversal of inhibitory effects of *Acanthamoeba castellanii* lysate for *Legionella pneumophila* using catalase. *Journal of Microbiological Methods* **18,** 311-316.

Costas, M., Edwards, S.W., Lloyd, D., Griffiths, A.J. & Turner, G. (1983) Restriction enzyme analysis of mitochondrial DNA of members of the genus

Acanthamoeba as an aid in taxonomy. *FEMS Microbiology Letters* **17,** 231-234.

Cote, M.A., Irvine, J.A., Rao, N.A. & Trousdale, M.D. (1991) Evaluation of the rabbit as a model of Acanthamoeba keratitis. *Reviews of Infectious Diseases* **13 (Suppl 5),** S443-S444.

Culbertson, C.G., Smith, J.W. & Minner, J.R. (1958) Acanthamoeba: observations on animal pathogenicity. *Science* **127,** 1506.

Cursons, R.T.M. & Brown, T.J. (1978) Use of cell cultures as an indicator of pathogenicity of free-living amoebae. *Journal of Clinical Pathology* **31,** 1-11.

Cursons, T.M., Brown, T.J., Keys, E.A., Moriarty, K.M. & Till, D. (1980) Immunity to pathogenic free-living amoebae: role of humoral antibody. *Infection and Immunity* **29,** 401-407.

De Jonckheere, J. & van de Voorde, H. (1977) Comparative study of six strains of *Naegleria* with special reference to nonpathogenic variants of *Naegleria fowleri*. *Journal of Protozoology* **24,** 304-309.

De Jonckheere, J.F. (1980) Growth characteristics, cytopathic effect in cell culture, and virulence in mice of 36 type strains belonging to 19 different *Acanthamoeba* spp. *Applied and Environmental Microbiology* **39,** 681-685.

De Jonckheere, J.F. (1987) Epidemiology.in: *Infectious Diseases. Color Atlas Monographs. I. Amphizoic Amoebae: Human Pathology,* ed. Rondanelli, E.G. Ch. 5, pp127-147. Pavia, Italy: Piccin Nuova Libraria.

De Jonckheere, J.F. (1991) Ecology of *Acanthamoeba*. *Reviews of Infectious Diseases* **13 (Suppl 5),** S385-S387.

Devonshire, P., Munro, F.A., Abernethy, C. & Clark, B.J. (1993) Microbial contamination of contact lens cases in the west of Scotland. *British Journal of Ophthalmology* **77,** 41-45.

Diaz, J., Osuna, A., Rosales, M.J., Cifuentes, J. & Mascaro, C. (1991) Sucker-like structures in two strains of *Acanthamoeba*: scanning electron microscopy study. *International Journal for Parasitology* **21,** 365-367.

Donzis, P.B., Mondino, B.J., Weissman, B.A. & Bruckner, D.A. (1989) Microbial analysis of contact lens care systems contaminated with *Acanthamoeba*. *American Journal of Ophthalmology* **108,** 53-56.

Dougherty, P.J., Binder, P.S., Mondino, B.J. & Glasgow, B.J. (1994) *Acanthamoeba* sclerokeratitis. *American Journal of Ophthalmology* **117,** 475-479.

Douglas, M. (1930) Notes on the classification of the amoeba found by Castellani in cultures of a yeast-like fungus. *The Journal of Tropical Medicine and Hygiene* **33,** 258-259.

Efron, N., Young, G. & Brennan N.A. (1989) Ocular surface temperature. *Current Eye Research* **8,** 901-906.

Epstein, R.J., Wilson, L.A., Visvesvara, G.S. & Plourde, E.G. Jr (1986) Rapid diagnosis of *Acanthamoeba* keratitis from corneal scrapings using indirect fluorescent antibody staining. *Archives of Ophthalmology* **104,** 1318-1321.

Ferrante, A. & Bates, E.J. (1988) Elastase in the pathogenic free-living amoebae *Naegleria* and *Acanthamoeba* spp. *Infection and Immunity* **56,** 3320-3321.

Ficker, L. (1988) Acanthamoeba keratitis - the quest for a better prognosis. *Eye* **2 (Suppl),** S37-S45.

Fischer, J.M.C., Peterson, C.A. & Bols, N.C. (1985) A new fluorescent test for cell vitality using calcofluor white M2R. *Stain Technology* **60,** 69-79.

Florakis, G.J., Folberg, R., Krachmer, J.H., Tse, D.T., Roussel, T.J. & Vrabec, M.P. (1988) Elevated corneal epithelial lines in *Acanthamoeba* keratitis. *Archives of Ophthalmology* **106,** 1202-1206.

Flores, B.M., Garcia, C.A., Stamm, W.E. & Torian, B.E. (1990). Differentiation of *Naegleria fowleri* from *Acanthamoeba* species by using monoclonal antibodies and flow cytometry. *Journal of Clinical Microbiology* **28,** 1999-2005.

Font, R.L., Tapert, M.J., Robinson, N.M., Osato, M.S. & Jones, D.B. (1981) An animal model of *Acanthamoeba* keratitis. *Investigative Ophthalmology &*

Visual Science **20 (Suppl),** 8.

Font, R.L., Tapert, M.J., Robinson, N.M., Visvesvara, G.S., Murphy, D. & Osato, M.S. (1982) An animal model of *Acanthamoeba* keratitis: further studies with emphasis on the early phase destruction of the trophozoites. *Investigative Ophthalmology & Visual Science* **22 (Suppl),** 163.

Fritsche, T.R., Gautom, R.K., Seyedirashti, S., Bergeron, D.L. & Lindquist, T.D. (1993) Occurrence of bacterial endosymbionts in *Acanthamoeba* spp. isolated from corneal and environmental specimens and contact lenses. *Journal of Clinical Microbiology* **31,** 1122-1126.

Fulford, D.E., Bradley, S.G. & Marciano-Cabral, F. (1985) Cytopathogenicity of *Naegleria fowleri* for cultured rat neuroblastoma cells. *Journal of Protozoology* **32,** 176-180.

Garner, A. (1988) Pathology of corneal acanthamoebic infection in: *The cornea: Transactions of the world congress on the cornea III,* ed. Dwight, H. Chapt 96, pp 535-539. New York: Cavanagh Raven Press Ltd.

Garner, A. (1993) Pathogenesis of *Acanthamoeba* keratitis: hypothesis based on a histological analysis of 30 cases. *British Journal of Ophthalmology* **77,** 366-370.

Gast, R.J. & Byers, T.J. (1995). Genus- and subgenus-specific oligonucleotide probes for *Acanthamoeba. Molecular and Biochemical Parasitology* **71,** 255-260.

Gast, R.J., Ledee, D.R., Fuerst, P.A. & Byers TJ (1996) Subgenus systematics of *Acanthamoeba*: four nuclear 18S rDNA sequence types. *Journal of Eukaryotic Microbiology* **43,** 498-504.

Gherardi, E., Gray, J., Stoker, M., Perryman, M. & Furlong, R. (1989) Purification of scatter factor, a fibroblast-derived basic protein that modulates epithelial interactions and movement. *Proceedings of the National Academy of Science* **86,** 5844-5848.

Gordon, V.R., Asem, E.K., Vodkin, M.H. & McLaughlin, G.L. (1993) Acanthamoeb*a* binds to extracellular matrix proteins in vitro. *Investigative*

Ophthalmology & Visual Science **34,** 658-662.

Gradus, M.S., Koenig, S.B., Hyndiuk, R.A. & DeCarlo J (1989) Filter-culture technique using amoeba saline transport medium for the noninvasive diagnosis of *Acanthamoeba* keratitis. *American Journal of Clinical Pathology* **92,** 682-685.

Griffin, J.L. (1972) Temperature tolerance of pathogenic and nonpathogenic free-living amoebas. *Science* **178,** 869-870.

Hadas, E. & Mazur, T. (1993a) Biochemical markers of pathogenicity and virulence of *Acanthamoeba* sp strains. *Parasitology Research* **79,** 696-698.

Hadas, E. & Mazur, T. (1993b) Proteolytic enzymes of pathogenic and non-pathogenic strains of Acanthamoeba spp. *Tropical Medicine and Parasitology* **44,** 197-200.

Hay, J., Kirkness, C.M., Seal, D.V. & Wright, P. (1994) Drug resistance and *Acanthamoeba* keratitis: the quest for alternative antiprotozoal chemotherapy. *Eye* **8,** 555-563.

He, Y., McCulley, J.P., Alizadeh, H., Pidherny, M., Mellon, J., Ubelaker, J.E., Stewart, G.L., Silvany, R.E. & Niederkorn, J.Y. (1992) A pig model of Acanthamoeba keratitis: transmission via contaminated contact lenses. *Investigative Ophthalmology & Visual Science* **33,** 126-133.

He, Y., Niederkorn, J.Y., McCulley, J.P., Stewart, G.L., Meyer, D.R., Silvany,R. & Dougherty, J. (1990) In vivo and in vitro collagenolytic activity of *Acanthamoeba castellanii. Investigative Ophthalmology & Visual Science* **31,** 2235-2240.

Heffler, K.F., Eckhardt, T.J., Reboli, A.C. & Stieritz, D. (1996) *Acanthamoeba* endophthalmitis in acquired immunodeficiency syndrome. *American Journal of Ophthalmology* **122,** 584-586.

Hsu, S.M., Raine, L. & Fanger, H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *Journal of Histochemistry and Cytochemistry* **29,** 577-580.

Illingworth, C.D. & Cook, S.D. (1998) *Acanthamoeba* keratitis. *Survey Of Ophthalmology* **42,** 493-508.

Illingworth, C.D., Cook, S.D., Karabatsas, C.H. & Easty, D.L. (1995) *Acanthamoeba* keratitis: risk factors and outcome. *British Journal of Ophthalmology* **79,** 1078-1082.

Impraim, C.C., Saiki, R.K., Erlich, H.A. & Teplitz, R.L. (1987) Analysis of DNA extracted from formalin-fixed paraffin-embedded tissues by enzymatic amplification and hybridisation with sequence-specific oligonucleotides. *Biochemical and Biophysical Research Communications* **142,** 710-716.

Insler, M.S., Portnoy, S.L., Beuerman, R.W. & Kaufman, H.E. (1988) Radial intrastromal lines in *Acanthamoeba* keratitis. *Archives of Ophthalmology* **106,** 883.

Jahnes, W.G., Fullmer, H.M. & Li C.P. (1957) Free-living amoebae as contaminants in monkey kidney tissue culture. *Proceedings of the Society for Experimental Biology and Medicine* **96,** 484-488.

Jakovljevich R. & Talis, B. (1969) Recovery of a hartmannelloid ameba in the purulent discharge from a human ear. *Journal of Protozoology* **16 (Suppl),** 36.

John, D.T. & John, R.A. (1994) Enhancement of virulence of *Naegleria fowleri* by growth in Vero-cell cultures. *Journal of Parasitology* **80,** 149-151.

John, D.T. (1993) Opportunistically pathogenic free-living amebae in: *Parasitic protozoa*, eds. Kreier, J.P. & Baker, J.R. 2nd edition, Volume 3, pp143-246. San Diego: Academic Press Inc.

John, T., Lin, J., Sahm, D. & Rockey, J.H. (1991) Effects of corticosteroids in experimental Acanthamoeba keratitis. *Reviews of Infectious Diseases* **13 (Suppl 5),** S440-S442.

Johns, K.J., Head, W.S., Parrish, C.M., Williams, T.E., Robinson, R.D. & O'Day, D.M. (1989) Examination of hydrophilic contact lenses with light microscopy to aid in the diagnosis of Acanthamoeba keratitis. *American Journal of Ophthalmology* **108,** 329-331.

Johns, K.J., Head, W.S., Robinson, R.D., Williams, T.E. & O'Day D.M. (1991)

Examination of the contact lens with light microscopy: an aid in diagnosis of Acanthamoeba keratitis. *Reviews of Infectious Diseases* **13 (Suppl 5),** S425.

Johns, K.J., O'Day, D.M. & Feman S.S. (1988) Chorioretinitis in the contralateral eye of a patient with *Acanthamoeba* keratitis. *Ophthalmology* **95,** 635-639.

Johns, K.J., O'Day, D.M., Head, W.S., Neff, R.J. & Elliot, J.H. (1987) Herpes simplex masquerade syndrome: Acanthamoeba keratitis. *Current Eye Research* **6,** 207-212.

Johnson, A.M., Fielke, R., Christy, P.E., Robinson, B. & Baverstock, P.R. (1990) Small subunit ribosomal RNA evolution in the genus *Acanthamoeba. Journal of General Microbiology* **136,** 1689-1698.

Jones, B.R., McGill, J.I. & Steele, A.D.McG. (1975a) Recurrent suppurative kerato-uveitis with loss of eye due to infection by *Acanthamoeba castellani. Transactions of the Ophthalmological Society of the United Kingdom* **95,** 210-213.

Jones, D.B., Visvesvara, G.S. & Robinson, N.M. (1975b) *Acanthamoeba polyphaga* keratitis and *Acanthamoeba* uveitis associated with fatal meningoencephalitis. *Transactions of the Ophthalmological Society of the United Kingdom* **95,** 221-232.

Karayianis, S.L., Genack, L.J., Lundergan, M.K. & Schumann, G.B. (1988) Cytologic diagnosis of Acanthamoebic keratitis. *Acta Cytologica* **32,** 491-494.

Kelly, L.D. & Mitra, D. (1993) Temperature sensitivity of *Acanthamoeba castellanii* and *polyphaga* corneal isolates. *Investigative Ophthalmology & Visual Science* **34,** 1319.

Kelly, L.D. (1992) Reevaluation of host corneal tissue 1955 to 1970: calcofluor white staining for occult acanthamoebic infection. *Annals of Ophthalmology* **24,** 345-346.

Kennedy, S.M., Devine, P., Hurley, C., Ooi, Y-S. & Collum, L.M.T. (1995) Corneal infection associated with *Hartmannella vermiformis* in contact-lens wearer. *The Lancet* **346,** 637-638.

Kerr, K.G. (1994) The rap on REP - PCR based typing systems. *Reviews in Medical Microbiology* **5,** 233-244.

Key, S.N., Green, W.R., Willaert, E., Stevens, A.R & Key, S.N. Jr (1980) Keratitis due to *Acanthamoeba castellani*. A clinicopathologic report. *Archives of Ophthalmology* **98,** 475-479.

Kilvington, S., Beeching, J.R. & White, D.G. (1991) Differentiation of *Acanthamoeba* strains from infected corneas and the environment by using restriction endonuclease digestion of whole-cell DNA. *Journal of Clinical Microbiology* **29,** 310-314.

Kinnear, F.B. & Hay, J. (1996) Brolene eye drops. *The Pharmaceutical Journal* **256,** 701.

Kinnear, F.B., Hay, J., Dutton, G.N. & Smith, H.V. (1995) Presumed ocular larva migrans presenting with features of diffuse unilateral subacute neuroretinitis. *British Journal of Ophthalmology* **79,** 1140-1141.

Kinnear, F.B., Hay, J., Montgomery, D. & Seal, D.V. (1996) Abuse of Brolene eye drops with putative corneal infection. *Journal of Infection* **32,** 172-174.

Kirkness, C.M., Hay, J., Seal, D.V. & Aitken, D. (1994) Acanthamoeba keratitis. *Ophthalmology Clinics of North America* **7,** 605-616.

Knox, D.L. & Bayless, T. (1975) Gastrointestinal and ocular disease in: *The Eye and systemic disease*, ed. Mausoff, F.A. Pp274-290. St Louis: Mosby.

Kwok, S. & Higuchi, R. (1989) Avoiding false positives with PCR. *Nature* **339,** 237-238.

Lai, S. & Henney, H.R. (1993) Nucleotide sequence of a portion of the 26S rDNA of *Acanthamoeba. Nucleic Acids Research* **21,** 4401.

Lai, S., Asgari, M. & Henney, H.R. Jr (1994) Non-radioactive DNA probe and polymerase chain reaction procedures for the specific detection of *Acanthamoeba. Molecular and Cellular Probes* **8,** 81-89.

Larkin, D.F.P. & Easty, D.L. (1990a) Experimental *Acanthamoeba* keratitis: I. Preliminary findings. *British Journal of Ophthalmology* **74,** 551-555.

Larkin, D.F.P. & Easty, D.L. (1991) Experimental *Acanthamoeba* keratitis: II Immunohistochemical evaluation. *British Journal of Ophthalmology* **75,** 421-424.

Larkin, D.F.P., Berry, M. & Easty, D.L. (1991) *In vitro* corneal pathogenicity of *Acanthamoeba*. *Eye* **5,** 560-568.

Larkin, D.F.P., Kilvington, S. & Dart, J.K.G. (1992) Treatment of *Acanthamoeba* keratitis with polyhexamethylene biguanide. *Ophthalmology* **99,** 185-191.

Larkin, D.F.P., Kilvington, S. & Easty, D.L. (1990) Contamination of contact lens storage cases by *Acanthamoeba* and bacteria. *British Journal of Ophthalmology* **74,** 133-135.

Ledee, D.R., Hay, J., Byers, T.J., Seal, D.V. & Kirkness, C.M. (1996) *Acanthamoeba griffini*: molecular characterization of a new corneal pathogen. *Investigative Ophthalmology & Visual Science* **37,** 544-550.

Lindquist, T.D. (1998) Treatment of *Acanthamoeba* keratitis. *Cornea* **17,** 11-16.

Lindquist, T.D., Fritsche, T.R. & Grutzmacher, R.D. (1990) Scleral ectasia secondary to *Acanthamoeba* keratitis. *Cornea* **9,** 74-76.

Luxenberg, M.N. (1990) Recurrent *Acanthamoeba* keratitis following penetrating keratoplasty. *Archives of Ophthalmology* **108,** 1482-1483.

Ma, P., Visvesvara, G.S., Martinez, A.J., Theodore, F.H., Daggett, P-M. & Sawyer, T.K. (1990) Naegleria and Acanthamoeba infections: review. *Reviews of Infectious Diseases* **12,** 490-513.

MacKay, R.M. & Doolittle, W.F. (1981) Nucleotide sequences of *Acanthamoeba castellanii* 5S and 5.8S ribosomal ribonucleic acids: phylogenetic and comparative structural analysis. *Nucleic Acids Research* **9,** 3321-3334.

Mannis, M.J., Tamaru, R., Roth, A.M., Burns, M. & Thirkill, C. (1986) *Acanthamoeba* scerokeratitis: determining diagnostic criteria. *Archives of Ophthalmology* **104,** 1313-1317.

Marciano-Cabral, F.M., Patterson, M., John, D.T. & Bradley, S.G. (1982) Cytopathogenicity of *Naegleria fowleri* and *Naegleria gruberi* for established mammalian cell cultures. *Journal of Parasitology* **68,** 1110-1116.

Marciano-Cabral, F., Zoghby, K.L. & Bradley, S.G. (1990) Cytopathic action of *Naegleria fowleri* amoebae on rat neuroblastoma target cells *Journal of Protozoology* **37,** 138-144.

Marciano-Cabral, F. & John, D.T. (1983) Cytopathogenicity of *Naegleria fowleri* for rat neuroblastoma cell cultures: scanning electron microscope study. *Infection & Immunity* **40,** 1214-1217.

Marciano-Cabral, F. & Toney, D.M. (1994) Modulation of biological functions of *Naegleria fowleri* amoebae by growth medium. *Journal of Eukaryotic Microbiology* **41,** 38-46.

Marciano-Cabral, F. (1988) Biology of *Naegleria* spp. *Microbiological Reviews* **52,** 114-133.

Marines, H.M., Osato, M.S. & Font, R.L. (1987) The value of calcofluor white in the diagnosis of mycotic and *Acanthamoeba* infections of the eye and ocular adnexa. *Ophthalmology* **94,** 23-26.

Martinez, A.J. & Janitschke, K. (1985) *Acanthamoeba*, an opportunistic microorganism: a review. *Infection* **13,** 251-256.

Martinez, A.J. (1973) Experimental *Naegleria* meningoencephalitis in mice. Penetration of the olfactory mucosal epithelium by Naegleria and pathologic changes produced: a light and electron microscope study. *Laboratory Investigation* **29,** 121-133.

Martinez, A.J. (1983) Free-living amoebae: pathogenic aspects. A review. *Protozoological Abstracts* **7,** 293-306.

Martinez, A.J. (1991) Infection of the central nervous system due to *Acanthamoeba*. *Reviews of Infectious Diseases* **13 (Suppl 5),** S399-S402.

Mathers, W., Stevens, G. Jr, Rodrigues, M., Chan, C.C., Gold, J., Visvesvara,G.S., Lemp, M.A. & Zimmerman, L.E. (1987) Immunopathology and electron microscopy of *Acanthamoeba* keratitis. *American Journal of*

Ophthalmology **103,** 626-635.

Matias, R., Schottelius, J., Raddatz, C.F. & Michel, R. (1991) Species identification and characterization of an *Acanthamoeba* strain from human cornea. *Parasitology Research* **77,** 469-474.

Mazur, T. & Hadas, E. (1994) The effect of the passages of *Acanthamoeba* strains through mice tissues on their virulence and its biochemical markers. *Parasitology Research* **80,** 431-434.

Mazur, T. & Jozwiak, M. (1993) Extracerebral Infections of *Acanthamoeba* spp. in mice. *Wiadomosci Parazytologiczne* **39,** 357-366.

Mazur, T., Hadas, E. & Iwanicka, I. (1995) The duration of the cyst stage and viability and virulence of Acanthamoeba isolates. *Tropical Medicine and Parasitology* **46,** 106-108.

McLaughlin, G.L., Stimac, J.E., Luke, J.M., Kuhlenschmidt, M.S., Vernon, R.A., Morton, L.D., Visvesvara, G.S. & Whiteley, H.E. (1991) Development of *Acanthamoeba*-cornea coincubation assays. *Reviews of Infectious Diseases* **13 (Suppl 5),** S397-S398.

McLellan, K. & Coster, D.J. (1987) Acanthamoebic keratitis diagnosed by paracentesis and biopsy and treated with propamidine. *British Journal of Ophthalmology* **71,** 734-736.

Milligan, S.M. & Band, R.N. (1988) Restriction endonuclease analysis of mitochondrial DNA as an aid in the taxonomy of *Naegleria* and *Vahlkampfia*. *Journal of Protozoology* **35,** 198-204.

Mitro, K., Bhagavathiammai, A., Shou, O-M., Bobbett, G., McKerrow, J.H., Chokshi, R., Chokshi, B. & James, E.R. (1994) Partial characterisation of the proteolytic secretions of *Acanthamoeba polyphaga*. *Experimental Parasitology* **78,** 377-385.

Moore, M.B., Ubelaker, J.E., Martin, J.H., Silvany, R., Dougherty, J.M., Meyer, D.R. & McCulley, J.P. (1991) In vitro penetration of human corneal epithelium by *Acanthamoeba castellanii*: a scanning and transmission electron microscope study. *Cornea* **10,** 291-298.

Moore, M.B., McCulley, J.P., Kaufman, H.E. & Robin, J.B. (1986) Radial keratoneuritis as a presenting sign in *Acanthamoeba* keratitis. *Ophthalmology* **93,** 1310-1315.

Moore, M.B., McCulley, J.P., Luckenbach, M., Gelender, H., Newton, C., McDonald, M.B. & Visvesvara, G.S. (1985) *Acanthamoeba* keratitis associated with soft contact lenses. *American Journal of Ophthalmology* **100,** 396-403.

Moore, M.B., McCulley, J.P., Newton, C., Cobo, L.M., Foulks, G.N., O'Day,D.M., Johns, K.J., Driebe, W.M., Wilson, L.A., Epstein, R.J. & Doughman, D.J. (1987) *Acanthamoeba* keratitis. A growing problem in soft and hard contact lens wearers. *Ophthalmology* **94,** 1654-1661.

Morton, L.D., McLaughlin, G.L. & Whiteley, H.E. (1991a) Adherence characteristics of three strains of *Acanthamoeba*. *Reviews of Infectious Diseases* **13 (Suppl 5),** S424.

Morton, L.D., McLaughlin, G.L. & Whiteley, H.E. (1991b) Effects of temperature, amoebic strain and carbohydrates on *Acanthamoeba* adherence to corneal epithelium in vitro. *Infection and Immunity* **59,** 3819-3822.

Nagington, J., Watson, P.G., Playfair, T. J., McGill, J., Jones, B.R. & Steele, A.D. McG. (1974) Amoebic infection of the eye. *The Lancet* **December 28,** 1537-1540.

Neff, R.J. (1957) Purification, axenic cultivation and description of a soil amoeba, *Acanthamoeba* sp. *Journal of Protozoology* **4,** 176-182.

Neff, R.J. (1958) Methods of purifying amoebae by migration on agar surfaces. *Journal of Protozoology* **5,** 226-231.

Newman, W., Hay, J., Browne, B. & Seal, D.V. (1997) *Acanthamoeba* as a 'transient' in the corneal scrape of a poorly compliant soft contact lens wearer with peripheral keratitis. *Eye* **11,** 937-938.

Niederkorn, J.Y., Ubelaker, J.E., McCulley, J.P., Stewart, G.L., Meyer, D.R., Mellon, J.A., Silvany, R.E., He, Y., Pidherney, M., Martin, J.H. & Alizadeh, H. (1992) Susceptibility of corneas from various animal species to in vitro binding and invasion by Acanthamoeba castellanii. *Investigative*

Ophthalmology & Visual Science **33,** 104-112.

Osato, M., Robinson, N., Wilhelmus, K. & Jones, D. (1986) Morphogenesis of *Acanthamoeba castellanii*: titration of the steroid effect (abstract). *Investigative Ophthalmology & Visual Science* **27 (Suppl),** 37.

Osato, M.S., Robinson, N.M., Wilhelmus, K.R. & Jones, D.B. (1991) In vitro evaluation of antimicrobial compounds for cysticidal activity against *Acanthamoeba*. *Reviews of Infectious Diseases* **13 (Suppl 5),** S431-S435.

Page, F.C. (1967a) Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. *Journal of Protozoology* **14,** 499-521.

Page, F.C. (1967b) Re-definition of the genus *Acanthamoeba* with descriptions of three species. *Journal of Protozoology* **14,** 709-724.

Page, F.C. (1985) The limax amoebae: comparative fine structure of the Hartmannellidae (Lobosea) and further comparisons with the Vahlkampfiidae (Heterolobosea). *Protistologica* **T. XXI, fasc,** 361-383.

Page, F.C. (1988) *A new key to freshwater and soil gymnamoebae with instructions for culture.* Cumbria, England: Freshwater Biological Association.

Panjwani, N., Zhao, Z., Baum, J., Hazlett, L.D. & Yang, Z. (1997) Acanthamoeba bind to rabbit corneal epithelium in vitro. *Investigative Ophthalmology & Visual Science* **38,** 1858-1864.

Panjwani, N., Zhao, Z., Baum, J., Pereira, M. & Zaidi, T. (1992) *Acanthamoeba* bind to glycolipids of rabbit corneal epithelium. *Infection and Immunity* **60,** 3460-3463.

Pasternak, J.J., Thompson, J.E., Schultz, T.M.G. & Zachariah, Z. (1970) A scanning electron microscope study of the encystment of *Acanthamoeba castellanii*. *Experimental Cell Research* **60,** 290-298.

Pellegrin, J.J., Ortega-Barria, E., Barza, M., Baum, J. & Pereira, M.E.A. (1991) Neuraminidase activity in Acanthamoeba species trophozoites and cysts. *Investigative Ophthalmology & Visual Science* **32,** 3061-3066.

Pels, E. & Schuchard, Y. (1983) Organ-culture preservation of human corneas. *Documenta Ophthalmologica* **56,** 147-153.

Pettit, D.A.D., Williamson, J., Cabral, G.A. & Marciano-Cabral, F. (1996) *In vitro* destruction of nerve cell cultures by *Acanthamoeba* spp.: A transmission and scanning electron microscopy study. *Journal of Parasitology* **82,** 769-777.

Pfister, D.R., Cameron, J.D., Krachmer, J.H. & Holland, E.J. (1996) Confocal microscopy findings of *Acanthamoeba* keratitis. *American Journal of Ophthalmology* **121,** 119-128.

Preston, T.M. & King, C.A. (1984) Amoeboid locomotion of *Acanthamoeba castellanii* with special reference to cell-substratum interactions. *Journal of General Microbiology* **130,** 2317-2323.

Pussard, M. & Pons, R. (1977) Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba* (Protozoa, amoebida) *Protistologica* **T XIII, fasc. 4,** 557-598.

Qureshi, M.N., Perez, A.A. II, Madayag, R.M. & Bottone, E.J. (1993) Inhibition of *Acanthamoeba* species by *Pseudomonas aeroginosa*: rationale for their selective exclusion in corneal ulcers and contact lens care systems. *Journal of Clinical Microbiology* **31,** 1908-1910.

Radford, C.F., Bacon, A.S., Dart, J.K.G. & Minassian, D.C. (1995) Risk factors for Acanthamoeba keratitis in contact lens users: a case-control study. *British Medical Journal* **310,** 1567-1570.

Robin, J.B., Chan, R. & Andersen, B.R. (1988) Rapid visualization of *Acanthamoeba* using fluorescein-conjugated lectins. *Archives of Ophthalmology* **106,** 1273-1276.

Robin, J.B., Chan, R., Rao, N.A., Sharma, S. & Srinivasan, M. (1989) Fluorescein-conjugated lectin visualization of fungi and Acanthamoebae in infectious keratitis. *Ophthalmology* **96,** 1198-1202.

Rodriguez-Zaragoza, S. (1994) Ecology of free-living amoebae. *Critical Reviews in Microbiology* **20,** 225-241.

Rowbotham, T.J. (1980) Preliminary report on the pathogenicity of *Legionella*

pneumophila for freshwater and soil amoebae. *Journal of Clinical Pathology* **33,** 1179-1183.

Sawyer, T.K. (1971) *Acanthamoeba griffini*, a new species of marine amoeba. *Journal of Protozoology* **18,** 650-654.

Schaegel, T.F. Jr & Culbertson, C.G. (1972) Experimental Hartmannella optic neuritis and uveitis. *Annals of Ophthalmology* **Feb,** 103-112.

Schaumberg, D.A., Snow, K.K. & Dana, M.R. (1998) The epidemic of *Acanthamoeba* keratitis: Where do we stand? *Cornea* **17,** 3-10.

Schuster, F.L. & Visvesvara, G.S. (1996) Axenic growth and drug sensitivity studies of *Balamuthia mandrillaris*, an agent of amebic meningoencephalitis in humans and other animals. *Journal of Clinical Microbiology* **34,** 385-388.

Seal, D.V., Hay, J., Kirkness, C.M., Morrell, A., Booth, A., Tullo, A., Ridgway, A. & Armstrong, M. (1996) Successful medical therapy of *Acanthamoeba* keratitis with topical chlorhexidine and propamidine. *Eye* **10,** 413-421.

Sharma, S., Srinivasan, M., & George, C. (1990) *Acanthamoeba* keratitis in non-contact lens wearers. *Archives of Ophthalmology* **108,** 676-678.

Shibata, D., Martin, W.J. & Arnheim, N. (1988) Analysis of DNA sequences in forty-year-old paraffin-embedded thin-tissue sections: a bridge between molecular biology and classical histology. *Cancer Research* **48,** 4564-4566.

Shimoyama, Y., Hirohashi, S., Hirano, S., Noguchi, M., Shimosato, Y., Takeichi, M. & Abe, O. (1989) Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. *Cancer Research* **49,** 2128-2133.

Silvany, R.E., Luckenbach, M.W. & Moore, M.B. (1987) The rapid detection of *Acanthamoeba* in paraffin-embedded sections of corneal tissue with calcofluor white. *Archives of Ophthalmology* **105,** 1366-1367.

Singh, S. & Sachdeva, M.P.S. (1994) *Acanthamoeba* keratitis. *British Medical Journal* **309,** 273.

Sison, J.P., Kemper, C.A., Loveless, M., McShane, D., Visvesvara, G.S. &

Doresinski, S.C. (1995) Disseminated Acanthamoeba infection in patients with AIDS: case reports and review. *Clinical Infectious Diseases* **20,** 1207-1216.

Sogin, M.L. (1989) Evolution of eukaryotic microorganisms and their small subunit ribosomal RNAs. *American Zoology* **29,** 487-499.

Stevens, A.R, & O'Dell, W.D. (1974) In vitro growth and virulence of *Acanthamoeba*. *Journal of Protozoology* **60,** 884-885.

Stevens, A.R., Kilpatrick, T., Willaert, E. & Capron, A. (1977) Serologic analyses of cell-surface antigens of *Acanthamoeba* spp. with plasma membrane antisera. *Journal of Protozoology* **24,** 316-324.

Stocker, F.W., Eiring, A., Georgiade, R. & Georgiade, N. (1958) A tissue culture technique for growing corneal epithelial, stromal, and endothelial cells seperately. *American Journal of Ophthalmology* **46 (Suppl),** 294-298.

Stoker, G.G. (1990) The polymerase chain reaction and infectious diseases: hopes and realities. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84,** 755-756 & 758.

Stothard, D.R., Schroeder-Diedrich, J.M., Awwad, M.H., Gast, R.J., Ledee, D.R., Rodriguez-Zarogoza, S., Dean, C.L., Fuerst, P.A. & Byers, T.J. (1998) The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. *Journal of Eukaryotic Microbiology* **45,** 45-54.

Stratford, M.P. & Griffiths, A.J. (1978) Variations in the properties and morphology of cysts of *Acanthamoeba castellanii*. *Journal of General Microbiology* **108,** 33-37.

Sutphin, J.E., Robinson, N.M., Wilhelmus, K.R. & Osato, M.S. (1986) Improved detection of oculomycoses using induced fluorescence with cellufluor. *Ophthalmology* **93,** 416-417.

Taylor, W.M., Pidherney, M.S. Alizadeh, H. & Niederkorn, J.Y. (1995) In vitro characterization of *Acanthamoeba castellanii* cytopathic effect. *Journal of Parasitology* **81,** 603-609.

Theodore, F.H., Jakobiec, F.A., Juechter, K.B., Ma, P., Troutman, R.C., Pang,

P.M. & Iwamoto, T. (1985) The diagnostic value of a ring infiltrate in acanthamoebic keratitis. *Ophthalmology* **92,** 1471-1479.

Thomas, P.A. & Kuriakose, T. (1990) Rapid detection of *Acanthamoeba* cysts in corneal scrapings by lactophenol cotton blue staining. *Archives of Ophthalmology* **108,** 168.

Tyndall, R.L., Willaert, E., Stevens, A. & Nicholson, A. (1979) Pathogenic and enzymatic characteristics of *Acanthamoeba* isolated from cultured tumor cells. *Protistologica* **T. XV, fasc. 1,** 17-22.

Ubelaker, J.E., Moore, M.B., Martin, J.H., Silveny, R., Dougherty, J.M., Meyer, D.R. & McCulley, J.P. (1991) In vitro intercellular adherence of *Acanthamoeba castellanii*: a scanning and transmission electron microscopy study. *Cornea* **10,** 299-304.

van Klink, F., Alizadeh, H., He, Y., Mellon, J.A., Silvany, R.E., McCulley, J.P. & Niederkorn, J.Y. (1993) The role of contact lenses, trauma and Langerhans cells in a Chinese hamster model of Acanthamoeba keratitis. *Investigative Ophthalmology & Visual Science* **34,** 1937-1944.

van Klink, F., Alizadeh, H., Stewart, G.L., Pidherny, M.S., Silvany, R.E., He, Y., McCulley, J.P. & Niederkorn, J.Y. (1992) Characterization and pathogenic potential of a soil isolate and an ocular isolate of *Acanthamoeba castellanii* in relation to *Acanthamoeba* keratitis. *Current Eye Research* **11,** 1207-1220.

van Klink, F., Taylor, W.M., Alizadeh, H., Jager, M.J., van Rooijgen, N. & Niederkorn, J.Y. (1996) The role of macrophages in *Acanthamoeba* keratitis. *Investigative Ophthalmology & Visual Science* **37,** 1271-1281.

Visvesvara, G.S. & Balamuth, W. (1975) Comparative studies on related free-living and pathogenic amebae with special reference to *Acanthamoeba*. *Journal of Protozoology* **22,** 245-256.

Visvesvara, G.S. & Leitch, G.J. (1998) Can *Acanthamoeba* serve as vector of microsporidia, the causal agent of corneal and disseminated microsporidiosis of humans? *Eighth International Conference on the Biology and Pathogenicity of Free-living Amoebae* **(Abstracts).**

Visvesvara, G.S. (1991) Classification of *Acanthamoeba*. *Reviews of Infectious Diseases* **13 (Suppl 5),** S369-S372.

Visvesvara, G.S. (1995) Pathogenic and opportunistic free-living amoebae. In *Manual of Clinical Microbiology* 6th Edition. Eds. Murray, P.R., Baron, E.J., Pfuller, M.A. Tenover, F.C. & Yolken, R.H. Ch. 105. Washington DC: ASM Press.

Visvesvara, G.S., Jones, D. & Robinson, N.M. (1975) Isolation, identification, and biological characterization of *Acanthamoeba polyphaga* from a human eye. *The American Journal of Tropical Medicine and Hygiene* **24,** 784-790.

Visvesvara, G.S., Martinez, A.J., Schuster, F.C., Leitch, G.J., Wallace, S.V., Sawyer, T.K. & Anderson, M. (1990) Leptomyxid ameba, a new agent of amebic meningoencephalitis in humans and animals. *Journal of Clinical Microbiology* **28,** 2750-2756.

Visvesvara, G.S., Schuster, F.L. & Martinez, A.J. (1993) *Balamuthia mandrillaris,* N.G., N. Sp., agent of amebic meningoencephalitis in humans and other animals. *Journal of Eukaryotic Microbiology* **40,** 504-514.

Vodkin, M.H., Howe, D.K., Visvesvara, G.S. & McLaughlin, G.L. (1992) Identification of *Acanthamoeba* at the generic and specific levels using the polymerase chain reaction. *Journal of Protozoology* **39,** 378-385.

Volkonsky, M. (1931) *Hartmannella castellanii* Douglas et classification des *Hartmannelles*. *Archives de Zoologie Experimentale et Generale* **72,** 317-339.

Wang, S.S. & Feldman, H.A. (1967) Isolation of Hartmannella species form human throats. *The New England Journal of Medicine* **30,** 1174-1179.

Warhurst, D.C. & Mann, P.G. (1988) *Acanthamoeba* keratitis. *British Medical Journal* **296,** 568.

Weekers, P.H. & van der Drift, C. (1993) Nitrogen metabolizing enzyme activities in the free-living soil amoebae *Acanthamoeba castellanii, Acanthamoeba polyphaga* and *Hartmannella vermiformis*. *Journal of Eukaryotic Microbiology* **40,** 251-254.

Wilhelmus, K.R., Osato, M.S., Font, R.L., Robinson, N.M. & Jones, D.B.

(1986) Rapid diagnosis of *Acanthamoeba* keratitis using calcofluor white. *Archives of Ophthalmology* **104,** 1309-1312.

Winchester, K., Mathers, W.D., Sulphin, J.E. & Daly, T.E. (1995) Diagnosis of *Acanthamoeba* keratitis in vivo with confocal microscopy. *Cornea 14,* 10-17

Wittner, M. & Rosenbaum, R.M. (1970) Role of bacteria in modifying virulence of *Entamoeba histolytica.* Studies of amebae from axenic cultures. *The American Journal of Tropical Medicine and Hygiene* **19,** 755-761.

Wright, P., Warhurst, D. & Jones, B.R. (1985) *Acanthamoeba* keratitis successfully treated medically. *British Journal of Ophthalmology* **69,** 778-782.

Wright, P.A. & Wynford-Thomas, D. (1990) Polymerase chain reaction: miracle or mirage? A critical review of its uses and limitations in diagnosis and research. *Journal of Pathology* **162,** 99-117.

Yagita, K. & Endo, T. (1990) Restriction enzyme analysis of mitochondrial DNA of *Acanthamoeba* strains in Japan. *Journal of Protozoology* **37,** 570-575.

Yagita, K., Matias, R.R., Yasuda, T., Natividad, F.F., Enriquez, G.L. & Endo, T. (1995) *Acanthamoeba* sp. from the Philippines: electron microscopy studies on naturally occurring bacterial endosymbionts. *Parasitology Research* **81,** 98-102.

Yoshida-Noro, C., Suzuki, N. & Takeichi, M. (1984) Molecular nature of the calcium-dependent cell-cell adhesion system in mouse teratocarcinoma and embryonic cells studied with a monoclonal antibody. *Developmental Biology* **101,** 19-27.

Addendum.

Bibliography : ~~Erratum~~

Aimard, L., Brasseur, P., Favennec, C., Perrine, D., Watt, L. & Brasseur, G. (1998) Amebic keratitis due to a mixed infection with *Acathamoeba* and *Hartmannella* species. *Clinical Infectious Diseases* **26,** 187-188.

Campos, M., Szerenyi, K., Lee, M., McDonnell, J.M., Lopez, P.F. & McDonnell, P.J. (1994) Keratocyte loss after corneal deepithelialization in primates and rabbits. *Archives of Ophthalmology* **112,** 254-260.

Culbertson, C.G., Smith, J.W., Cohen, H.K. & Minner, J.R. (1959) Experimental infection of mice and monkeys by Acanthamoeba. *American Journal of Pathology* **35,** 185-197.

De Jonckheere, J.F. & Brown, S. (1998a) Is the free-living ameba *Hartmannella* causing keratitis? *Clinical Infectious Diseases* **27,** 1337-1338.

De Jonckheere, J.F. & Brown, S. (1998b) There is no evidence that the free-living ameba *Hartmannella* is a human parasite. *Clinical Infectious Diseases* **26,** 773.

De Jonckheere, J.F. & Brown, S. (1998c) Non-*Acanthamoeba* amoebic infection. *Journal of Infection* **36,** 349-350.

Inoue, T., Asari, S., Tahara, K., Hayashi, K., Kiritoshi, A. & Shimomura, Y. (1998) *Acanthamoeba* keratitis with symbiosis of *Hartmannella* ameba. *American Journal of Ophthalmology* **125,** 721-723.

Larkin, D.F.P. & Easty, D.L. (1990b) Exernal eye flora as a nutrient source for *Acanthamoeba*. *Graefe's Archive for Clinical and Experimental Ophthalmology* **228,** 458-460.

Larkin, D.F.P. & Leeming, J.P. (1991) Quantitative alterations of the commensal eye bacteria in contact lens wear. *Eye* **5,** 70-74.

Wilson, S.E., He, Y-G., Weng, J., Li, Q., McDowall, A.W., Vital, M. & Chwang, E.L. (1996) Epithelial injury induce keratocyte apoptosis: Hypothesized role for the Interleukin-1 system in the modulation of corneal tissue organization and wound healing. *Experimental Eye Research* **62,** 325-337.

APPENDIX A

Amoebal Culture: Methods and Reagents

Non-nutrient Agar

Agar bacteriological (Oxoid), at a concentration of 1% in distilled water, was autoclaved at 121 ^{0}C for 20 min.

Heat-killed Bacteria

A suspension of *Klebsiella pneumoniae*, in sterile normal saline, was autoclaved at 121 ^{0}C for 20 min.

Amoebal Saline

Amoebal saline was prepared by taking 5 ml each of No 1 and No 2 Stock solutions as detailed below, and making up to 1 L with distilled water.

No 1 Stock:

NaCl (BDH)	12.0	g
$MgSO_4$. $7H_2O$ (BDH)	0.4	g
$CaCl_2$. $6H_2O$ (BDH)	0.6	g
Distilled water	500	ml

No 2 Stock:

Na_2HPO_4 (BDH)	14.2	g
KH_2PO_4 (BDH)	13.6	g
Distilled water	500	ml

Proteose peptone-Yeast extract-Glucose (PYG) Medium

Proteose peptone-Yeast extract-Glucose (PYG) medium was prepared by dissolving all the ingredients listed below, except $CaCl_2$, in about 900 ml of distilled water. The $CaCl_2$ was then added while stirring, and the volume adjusted to 1000 ml. The resulting medium was autoclaved for 15 min, supplemented with penicillin and streptomycin at a final concentration of 400 U/ml and 400 µg/ml respectively and the pH adjusted to 6.5.

Proteose peptone (Difco)	20.0	g
Yeast extract (Difco)	2.0	g
$MgSO_4.7H_2O$	0.980	g
$CaCl_2$	0.059	g
Sodium citrate.$2H_2O$	1.0	g
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.02	g
KH_2PO_4	0.34	g
$Na_2HPO_4.7H_2O$	0.355	g
Glucose	18.0	g
Distilled water to	1.0	L

Proteose peptone-Glucose Medium

Proteose peptone (Difco)	15	g
Glucose (Sigma)	18	g
Amoebal saline	1.0	L

Proteose peptone-Yeast extract-Glucose-Serum Medium

Proteose peptone (Difco)	7.5	g
Yeast extract (Difco)	3.75	g
Glucose (Sigma)	9.0	g
Na_3PO_4 (BDH)	0.19	g
Foetal calf serum (FCS Gibco-BRL)	50	ml
Distilled water to	500	ml

Modified Chang's Medium

Casein digest (Difco)	10.0	g
Na_2HPO_4 (BDH)	2.5	g
KH_2PO_4 (BDH)	0.8	g
Yeast extract (Difco)	5.0	g
Glucose (Sigma)	2.5	g
Liver digest (Difco)	2.5	g
FCS (Gibco-BRL)	100	ml
Distilled water to	1.0	L

Neff's Optimal Growth Medium

Proteose peptone (Difco)	7.5	g
Yeast extract (Difco)	7.5	g
Glucose (Sigma)	15.0	g
$MgSO_4$ (BDH)	0.246	g
$CaCl_2$ (BDH)	0.006	g

KH_2PO_4 (BDH)	0.348	g
Ferric citrate (Sigma)	0.245	g
B_1 hydrochloride (Sigma)	1.0	µg
Biotin (Sigma)	0.2	mg
B_{12} (Sigma)	1.0	µg
Distilled water to	1.0	L

PYNFH Medium

Proteose peptone (Difco)	10.0	g
Yeast extract (Difco)	10.0	g
Yeast nucleic acid (Sigma)	1.0	g
Folic acid (Sigma)	15	mg
Hemin (Sigma)	1	mg
Buffer (see below)	20	ml
FCS (Gibco-BRL)	100	ml
Distilled water	1.0	L

Buffer: Na_2HPO_4 (25 g) and KH_2PO_4 (18.1 g), made up to 1 L with distilled water.

Balamuthia medium

Biosate peptone (BBL)	2.0	g
Yeast extract (Difco)	2.0	g
Torula yeast RNA (Sigma)	0.5	g

Hanks balanced salts, 10X (Gibco-BRL)	34.0	ml
5% ox liver digest in Hanks salts (Panmede)	100.0	ml
MEM vitamin mixture 100X (Sigma)	5.0	ml
MEM nonessential amino acid 100X (Sigma)	5.0	ml
Lipid mixture 1000X (Sigma)	0.5	ml
10% glucose	5.0	ml
Hemin at 2g/ml (Sigma)	0.5	ml
0.5% taurine (Sigma)	5.0	ml
FCS (Gibco-BRL)	50.0	ml
Distilled water to	500.0	ml

FKMx4 Medium

A mixture of equal volumes of PYNFH, *Balamuthia*, PYG and Neff's optimal growth media.

APPENDIX B

Cell Culture: Methods and Reagents

Indirect Alkaline Phosphatase Protocol

Coverslips of cultured cells were treated as follows, where the primary antibody was mouse anti-fibroblast (DAKO; 1:00) and the secondary antibody was rabbit anti-mouse alkaline phophatase conjugate (DAKO; 1:30).

1. Fixed in neutral buffered formalin.

2. Washed twice in phosphate buffered saline (PBS - 5 min).

3. Incubated with primary antibody (1 hr).

4. Washed twice in PBS (5 min).

5. Incubated with secondary antibody (1 hr).

6. Visualised with Vector Red Alkaline Phosphatase Substrate Kit (Vector Laboratories).

8. Washed twice in PBS (5 min).

9. Mounted in synthetic resin.

Indirect Immunofluorescence

Coverslips of cultured cells were treated as follows, where the primary antibody was mouse anti-human pancytokeratin (Sigma; 1:100) and the secondary antibody was sheep anti-mouse IgG labelled with Texas Red (Amersham LIFE SCIENCE; 1:100):

1. Fixed and permeabilised by adding 1 ml of methanol/acetic acid (3:1) for 5 min, without removing the growth medium, and then replacing with 2 ml fresh methanol/acetic acid for a further 5 min.

2. Washed twice in PBS (5 min).

3. Incubated with primary antibody (30 min).

4. Washed twice in PBS (5 min).

5. Incubated with secondary antibody (30 min).

6. Washed twice in PBS (5 min) and mounted in PBS/glycerol.

Endothelial Cell Growth Medium

Endothelial cell basal medium (unsupplemented MCDB 131, Clonetics catalogue no CC-3121) with endothelial cell growth medium bullet kit (Clonetics catalogue no CC-3124) containing single use aliquots of epidermal growth factor (10 ng/ml), hydrocortisone (1 μg/ml), bovine brain extract, foetal bovine serum (2%), gentamicin 50 μg/ml and amphotericin-B 50 ng/ml.

HEPES Saline

HEPES stands for N-2-hydroxyethylpiperazine N'-2-ethane sulfuric acid. HEPES saline was prepared from the following, pH adjusted to 7.5 % with 5 M NaOH, then aliquoted, autoclaved and stored at 4 ^{0}C.

NaCl (BDH)	140 mM
KCl (BDH)	5 mM
d-glucose (BDH)	5 mM
HEPES (Sigma)	10 mM
0.5% phenol red	0.001%

Trypsin/versene

Trypsin (2.5%, GibcoBRL) diluted to 0.25% vol/vol in versene 1:5000 (GibcoBRL).

Freeze Mix

2% dimethylsulphoxide (Sigma) in foetal calf serum (FCS - GibcoBRL), filtered to sterilise.

APPENDIX C

Histology and Immunodiagnosis: Methods and Reagents

Differential Isolation of White Blood Cells

Polymorphs and lymphocytes were separated as follows:

1. 20 ml of heparinised blood was obtained from a volunteer by venepuncture.

2. An equal volume of PBS was added.

3. PBS/blood mixture was aliquoted onto lymphoprep (sodium metrozoate/ficoll sg 1.077; NYCOMED Ltd) in the ratio 4 ml diluted blood to 3 ml lymhoprep and allowed to stand for 30 min in order to allow red blood cell aggregation.

4. PBS/blood/lymhoprep mixture was then centrifuged at 400 g for 30 min. This produced a pellet of red blood cells and polymorphs with lymphocytes and monocytes at the plasma/ficoll interface.

Lymphocytes and monocytes:

1. Supernatant containing mononuclear cells was aspirated and washed at 150 g for 30 min.

2. Resulting pellet was resuspended in an equal volume of growth medium supplemented with FCS and washed at 200 g for 15 min.

3. Resulting pellet was resuspended in 2 ml of growth medium supplemented with FCS and adjusted to 1×10^6 cells/ml for cytospin preparations.

Red blood cells and polymorphs:

1. Pellet was resuspended in an equal volume of autologous plasma.

2. Dextran 6% in PBS (4 ml per 20 m of blood) was added. and allowed to

sediment until approximately half way down the gradient (30 min - 1 hr).

3. Supernatant was then pipetted off.

4. Red blood cells and polymorphs were pelleted by centrifugation at 200g.

5. Pellet was resuspended in 1 ml distilled H_2O for 10 sec to produce lysis of the red cells then 1ml of 2X PBS added.

6. The polymorphs were then pelleted at 200 g, washed once in 1X PBS, repelleted and adjusted to 1 x 10^6 cells/ml for cytospin preparations.

APES-Coated Microscope Slides

Slides were coated with 2% 3-aminopropyltriethoxysilane (APES, Sigma) as follows:

1. Washed in 1% Triton X 100 (Sigma).

2. Rinsed in running tap water.

3. Immersed in 2% APES in acetone for 5 min.

4. Washed in tap water for 5 min.

5. Air dried overnight and reboxed.

Calcofluor White

Calcofluor white staining of sections was carried out according to the following protocol:

1. Paraffin wax-embedded sections, but not cytospin preparations, were dewaxed in xylene (5 min) and taken through graded alcohols to water.

2. Sections were stained for 5 min with freshly prepared 0.1% calcofluor white (Sigma) and 0.1% Evan's blue (Sigma) in distilled water.

3. Sections were then drained and mounted (Uvinert mountant, Searle Scientific Services).

Note: The effects of trypsin digestion prior to staining was assessed for paraffin-wax embedded sections using 0.1% in PBS, pH 7.8 for 10 min at 37 ^{0}C or 0.25% for 30 min)

Indirect Immunofluorescence Protocol

The immunofluorescence labelling method comprised of the following steps:

1. Fixation with acetone.

2. Washing with Tris buffered saline (TBS) - pH 7.6.

3. Blocking of non-specific binding with normal swine serum (NSS; DAKO - diluted 1:5 in TBS) for 5 min.

4. Washing with TBS and removal of excess buffer.

3. Incubation with the primary antibody diluted in 1:25 NSS in TBS for 30 min.

4. Washing in TBS with agitation for 2 periods of 5 min each, then removal of excess buffer.

5. Incubation with the secondary antibody comprising fluorescein-isothiocyanate-labelled swine anti-rabbit immunoglobulins (diluted 1:20 in 1:25 NSS in TBS) for 30 min.

6. Washing in TBS with agitation for 2 periods of 5 min each, then removal of excess buffer.

9. Mounting in Uvinert mountant (Searle Scientific Services).

Indirect Alkaline Phosphatase Protocol

The same method as described in Appendix B was used for cytospin preparations of amoebae, except that the primary antibody was SK anti-*Acanthamoeba* (1:1000) and the secondary antibody was swine anti-rabbit alkaline phosphatase conjugate.

ABC-Peroxidase Immunolabelling Protocol

The ABC-Peroxidase immunolabelling method was carried out by treating paraffin wax-embedded sections as detailed below:

1. Washed in xylene (2x5 min) then passed through 3 changes of absolute alcohol.

2. Rinsed with methanol.

3. 3% hydrogen peroxide for 30 min.

4. Washed in water.

5. If the specimen had originally been glutaraldehyde-fixed it was digested with 0.1% trypsin and 0.1% calcium chloride dissolved in TBS at 37 ^{0}C (10 min) in order to deprotect the antigenic sites; this was not required for formalin-fixed specimens.

6. Washed in water/buffer.

7. Incubated for 10 min with 1:5 normal goat serum (NGS, SAPU) in TBS in order to block non-specific binding.

8. Incubated with primary antibody diluted in 1:25 NGS in TBS for either 18 hr overnight at 4 ^{0}C or 30-60 min at room temperature.

9. Washed with TBS - 3x10 min.

10. Incubated with DAKO DUET ABC anti-mouse anti-rabbit as per instructions (i.e. secondary antibody, biotinylated goat anti-rabbit, diluted 1:200 in 1:250 NGS in TBS and tertiary antibody, ABC-Peroxidase at 1:100).

11. Washed with TBS 3x10 min.

14 Visualised with di-aminobenzidine (DAB).

15 Washed in water.

16. Stained lightly with Haematoxylin/red counterstain.

17. Rehydrated in graded alcohols, cleared in xylene and mounted in resin.

APPENDIX D

Molecular Biology: Methods and Reagent

Proteinase K Digest Solution

Proteinase K (Promega) was prepared, from 10 mg/ml stock, at 500 µg/ml in proteinase K buffer, then aliquoted and stored frozen at - 20 ^{0}C.

Proteinase K buffer:

50 mM Tris HCl pH8.5 (Sigma)

1 mM EDTA (Sigma)

0.5% TWEEN (Sigma)

'Cold' Polymerase Chain Reaction (PCR) Protocol

1) After preparation of the dNTP mix, a master mix was prepared containing the following per 50 µl reaction volume:-

1ul of each primer

5 µl of 10X buffer (GibcoBRL)

3 µl of $MgCl_2$ (GibcoBRL)

8 µl of dNTP mix (GibcoBRL)

28.5 µl of nonpyogenic DEPC/H_2O

2.5 µl of 1% W1 detergent (GibcoBRL)

2) 1 µl of sample was added to a 49 µl aliquot of the master mix and layered with mineral oil (Sigma) to prevent evaporation. After a hot start (i.e. heating to 93 ^{0}C for 2 min then holding at 90 ^{0}C), 0.25 µl of *Taq* DNA polymerase (Promega) was added to each sample.

3) Amplifications were performed for 35 cycles of: 93 ^{0}C for 1 min; 61^0 C for 1 min; 72 ^{0}C for 2 min; followed by a final elongation step of 72 ^{0}C for 10 min.

4) Aliquots of the PCR products (10 µl in 4 µl of loading dye 1), along with 4 µl of diluted 1 Kb ladder (GibcoBRL), were run at 80 V for 1 hr on a standard 2% agarose gel containing ethidium bromide.

TBE Buffer:

100 mM Tris base (Sigma)

90 mM boric acid (Sigma)

1 mM EDTA (Sigma) (20 ml 0.5 M pH 8)

Loading Dye 1:

0.25% bromophenol blue (Sigma)

40% glycerol (BDH)

0.1% orange G (BDH)

Agarose Gel:

2% agarose (GibcoBRL) in TBE buffer, supplemented (per 50 ml gel) with 0.4 ul of 10 mg/ml ethidium bromide (Sigma) in DEPC/H_2O

DEPC/H_2O and DEPC/PBS:

0.1% Diethylpyrocarbonate in distilled water or in PBS, incubated for 24 hours at 37 ^{0}C, then autoclaved.

dNTPS Mix:

For 'cold' PCR: total concentration of 1.25 mM in DEPC H_2O, prepared with

equal amounts of dATP, dCTP, dGTP, dTTP (Promega).

'Hot' PCR

"Hot' PCR was performed as for 'cold' PCR, except that the dNTP mix contained only 10% of the amount of dCTP, which was then supplemented by adding 0.1 μci (0.1 μl) of 5" P32 dCTP (Amersham LIFE SCIENCE) per 25 μl reaction volume to the master mix. Also the resulting product was loaded as 2 μl of sample in 2 μl of loading dye, and run on a polyacrylamide gel (prepared and run as described for 'sequencing gel' in Appendix D).

Loading Dye 2:

95% deionised formamide (Sigma)

0.2% bromophenol blue (Sigma)

Southern Blotting (SB) Hybridisation Protocol

1. Hybond N blot was placed in a roller tube containing 6 ml of standard SB Prehybridisation Buffer and incubated at 52 ^{0}C (anticipated hybridisation temperature) for at least 1 hr. (The anticipated hybridisation temperature was derived from the formula $T_{max} = 81.5 + 16.6 \log M - 0.72 F + 0.41 (\% G = C) - 650/L$ where M = cation concentration, F = formamide and L = no of bases.)

2. The Prehybridisation Buffer was discarded, and replaced with 5 ml of Hybridisation Solution (labelled probe diluted in standard SB Prehybrisation Buffer to the desired concentration of 0.1 - 2 pmol/ml eg 20 μl in 5 ml) and allowed to hybridise at 52 ^{0}C overnight.

3. The Hybridisation Solution was discarded and the blot washed twice (5 min per wash) in SB 2X Wash Solution at 52 ^{0}C followed by two 15 min washes in SB 0.1X Wash Solution at 52 ^{0}C.

4. The blot was incubated in SB Buffer 2 for 30 min.

5. The blot was incubated in anti-digoxigenin alkaline phosphatase-conjugated antibody (Boehringer Mannheim) diluted 1:5000 in SB Buffer 2.

6. The blot was washed twice, 15 min per wash, in SB Buffer 1.

7. The blot was incubated in SB Buffer 3 for 2 min in order to activate the alkaline phosphatase.

8. Colour substrate solution was freshly prepared from 45 µl nitrobluetetrazolium (Sigma) and 35 µl bromochloroindolyl phosphate (Sigma) solution in 10 ml of SB Buffer 3.

9. SB Buffer 3 was decanted, the colour substrate solution added and the colour development allowed to proceed in the dark. (Colour precipitate starts to form within a few minutes and the reaction is usually complete within 16 hr.)

10. When bands of the desired intensity develop, the reaction was terminated by washing the membrane for 5 min with 50 ml of SB Buffer 4.

SB Denaturing Solution:

0.5 N NaOH (Sigma) and 1.5 M NaCl (Sigma).

SB Neutralising Solution:

0.5 M Tris-HCl (Sigma) and 3 M NaCl (Sigma); pH 7.5.

20X SCC Buffer:

3 M NaCl (Sigma) and 300 mM sodium citrate (Sigma); pH 7.0.

SB Prehybridisation Buffer:

5X SSC Buffer with 1.0% (w/v) blocking reagent for nucleic acid hybridisation (Boehringer Mannheim), 0.1% N-lauroylsarcosine (Sigma), 0.02% sodium dodecyl sulfate (ICN).

SB Hybridisation Buffer:

DIG-labelled probe diluted in standard SB Prehybridisation Buffer.

SB 2X Wash Solution:

2X SCC containing 0.1% sodium dodecyl sulfate (ICN).

SB 0.1X Wash Solution:

0.1X SCC containing 0.1% sodium dodecyl sulfate (ICN).

SB Buffer 1:

100 mM Tris HCl (Sigma) and 150 mM NaCl (Sigma); pH 7.5.

SB Buffer 2:

As Buffer 1 with 1 g/100 ml of blocking reagent for nucleic acid hybridisation and detection (Boehringer Mannheim).

SB Buffer 3:

100 mM Tris (Sigma), 100 mM NaCl (Sigma) and 50 mM $MgCl_2$ (Sigma); pH 9.5.

SB Buffer 4:

70 mM Tris HCl (Sigma) and 1 mM EDTA (Sigma); pH 8.

DNA Probe Labelling

The 126-bp probe ArDNA-a, complementary to the expected PCR product, was labelled at the 3' end with digoxigenin-11-dUTP/dATP using the Boehringer Mannheim DIG Oligonucleotide Tailing Kit according to the following protocol:-

1) The reagents listed below were added to a sterile microcentrifuge tube (on ice) in the following order:-

5X reaction buffer with $CoCl_2$	4 µl
Digoxigenin-11-dUTP	1 µl
Oligonucleotide	x µl (variable - approx 100 pmol)
dATP	1 µl
Terminal Transferase	1 µl
H_2O	x µl (to adjust total volume to 20 µl)

2. The reaction was incubated at 37 ^{0}C for 15 min and placed on ice.

3. One µl of glycogen solution and 1 µl of EDTA were added to the reaction tube.

4. The labelled oligonucleotide were precipitated with 0.1 volume (2.5 µl) of 4 M LiCl and 2.5-3.0 volumes (75 µl) of pre-chilled ethanol. This was mixed well and incubated at -70 ^{0}C for at least 30 min or at -20 ^{0}C for 2 hr.

5. The reaction was centrifuged at 13 000 g for 15 min in a microcentrifuge at 4 ^{0}C.

6. The first ethanol wash was decanted and the pellet rewashed with 50 µl of cold 70% ethanol and centrifuged at 13 000 g for 5 min at 4 ^{0}C.

7. The second ethanol wash was decanted. The pellet was dried and resuspended in 20 µl of sterile double distilled water.

The quality of the labelling obtained was estimated by comparing the density of colour precipitate produced by the intended visualisation procedure for southern blotting, compared to that obtained with serial dilutions of a labelled

control probe of known concentration. The labelled probe may be stored at -20 ^{0}C if not used immediately.

Cloning

Cloning was performed as detailed in Section 2.2.2.4 using the pMOS*Blue* T-vector Kit RPN 1719 (Amersham LIFE SCIENCE).

Ligation Mixture:

10X ligase buffer	1.0 µl
100 mM DTT	0.5 µl
10 mM ATP	0.5 µl
50 ng/ul vector	1.0 µl
PCR product (insert)	Appropriate amount (up to 2 µl)
T4 DNA ligase	0.5 µl
DEPC/H_2O	x µl (to adjust total volume to 10 µl)

Tryptone-Yeast Medium (per L):

10 g tryptone (Difco)

5 g yeast extract (Difco)

10 g NaCl (Sigma)

L-agar plates (per L):

10 g tryptone (Difco)

5 g yeast extract (Difco)

10 g NaCl (Sigma)

15 g agar (Difco)

Sequencing

Sequencing was performed as described in Section 2.2.2.5 using the Sequenase Version 2.0 DNA Sequencing Kit (Amersham LIFE SCIENCE).

Annealing Mixture:

DNA	up to 7 µl containing 3-5 mg (for plasmid)
H_2O	x µl (to adjust total volume to 10 µl)
Reaction buffer	2 µl
Primer	1 µl of a PCR primer diluted 1:5 (in DEPC/H_2O)

Labelling Mixture:

Ice cold annealed DNA mixture	10 µl
Dithiothreitol (DTT) 0.1M	1 µl
Diluted Labelling Mix	2 µl
[^{35}S dATP]	0.5 µl
Diluted Sequenase Polymerase	2 µl

[NB: Labelling Mix was diluted 1:4 in DEPC/H_2O and Sequenase DNA Polymerase was diluted 1:8 in ice cold enzyme dilution buffer, immediately before use.]

Sequencing Gel Protocol:

1. 25 ml acrylamide ("Easigel", from Scotlab) and 30 ml of 5X TBE buffer were added to a 200 ml glass beaker containing 63 g urea (Fisons).

2. The gel solution was made up to 150 ml with deionised water and mixed on a magnetic stirrer until dissolved.

3. 10 ml of the solution were removed and 75 µl of Temed (N, N, N', N'-tetramethylethylenediamine, from Biorad) and 75 µl from 1 ml of 10% ammonium persulphate (Biorad) were added. The solution was mixed and used to make a two cm gel plug along the bottom end of the gel plates, using a 2 ml syringe.

4. 75 µl of Temed were added to the remainder of the gel mixture, followed by the remainder of the ammonium persulphate. The solution was mixed and loaded between the gel plates with a 50 ml syringe.

5. Once the gel was poured, the combs were placed in position, upside down along the top edge of the gel, to form a straight edge.

6. Approximately one hour after pouring the gel, a piece of absorbent paper soaked in 0.5X TBE buffer was placed at either end of the gel plates and covered with cling film. The gel was left overnight at room temperature to set.

7. 1500 ml of 0.5X TBE buffer were prepared and heated at full power in a microwave, for 8 min. A further 450 ml of cold 0.5X TBE buffer was added to the base of the gel rig.

8. The combs were removed from the set gel, the heated buffer was poured between the gel plates, and the gel was pre-run at 95 Watts until the temperature reached 50 ^{0}C . At this point the combs were placed into the gel, right way up, and 4 µl of each sample (in Sequenase Kit Stop Solution) were loaded onto the gel along with a sequencing ladder.

9. The gel was run, maintaining a temperature of 50 ^{0}C, until the bromophenol blue dye front had almost reached the bottom of the gel. The plates were then removed from the rig and the back plate was carefully removed.

10. A sheet of Whatman chromatography paper was smoothed onto the gel then gently removed, along with the attached gel.

11. Gels were dried, for 2 hr at 80 ^{0}C, on a gel drier (Biorad) and an autoradiograph was taken of the dried gel.

NB: Before use gel plates were cleaned with 10% Decon (Decon Laboratories Ltd), rinsed well with tap water and then with ethanol. The back plate only was

treated with Repelcote (BDH) for thirty seconds, followed by gentle polishing.

Sequencing Ladder:

The sequencing ladder was prepared by using Sequenase Version 2.0 DNA Sequencing Kit (Amersham LIFE SCIENCE) and Sequencing primer -40 to sequence Control ssDNA M13mp18.

In Situ Hybridisation (ISH) Method

Paraffin wax-embedded sections were treated as follows:

1. Dewaxed with xylene (5 min), then rehydrated by immersion in alcohol (3 min), followed by spirit (3 min), then DEPC/PBS.

2 Immersed in 0.2N HCl for 15 min. (60 ml 1N HCl (Sigma) + 240 ml DEPC/H_2O) and rinsed in DEPC/PBS.

3. Immersed in 0.3% Triton X (Sigma) for 15 min (900 µl + 300 ml DEPC/PBS) and rinsed in DEPC/PBS.

4. Digested with proteinase K (100 µg/ml, dilute in DEPC/PBS from 1 mg/ml stock, Promega) for 30 min at 37 ^{0}C, then rinsed in DEPC/PBS.

5. Fixed in 2% paraformaldehyde for 5 min (6 g in 300 ml DEPC/PBS) and rinsed in DEPC/PBS.

6. Incubated in ISH Prehybridisation Buffer for 2 hr at 37 ^{0}C.

7. Hybridised overnight at 42 ^{0}C with probe which has been diluted 1:20 in ISH hybridisation buffer, then heated at 70 ^{0}C for 2 min and quenched on ice immediately prior to application.

NB The above method refers to paraffin wax-embedded sections. For cytospin preparations, step 1 was omitted and incubation times in steps 2-4 reduced by two thirds.

ISH Visualisation Stage :

Following hybridisation, the slides were treated as follows to detect the presence of labelled-probe-target complexes:

1. Washed in 2X SSC for 30 min at room temperature.

2. Washed in 2X SSC for 30 min at room temperature.

3. Washed in 0.1X SSC for 10 min at room temperature.

4. Washed in 0.1X SSC for 30 min at 50 ^{0}C.

5. Washed in 0.1X SSC for 20 min at room temperature.

6. Immersed in Digoxigenin Buffer 1 for 5 min at room temperature.

7. Incubated with rabbit serum (SAPU-diluted 1:5 in Digoxigenin Buffer 1) for 10 min

8. Incubated in mouse monoclonal anti-digoxin (Sigma, diluted 1:7000 in Digoxigenin Buffer 1) for 30 min

9. Washed in Digoxigenin Buffer 1 for 5 min, twice

10. Incubated with rabbit antimouse (DAKO, 1:60 in Digoxigenin Buffer 1 with 10% normal human serum) for 30 min.

11. Washed in Digoxigenin Buffer 1 for 5 min, twice

12. Incubated with alkaline phosphatase-conjugated mouse anti-alkaline phosphatase (DAKO, 1:90 in Digoxigenin Buffer 1) for 30 min.

13. Washed in Digoxigenin Buffer 1 for 5 min, twice.

14. Steps 10-13 were repeated, but incubating with each antibody for only 10 min.

15. Washed with Digoxigenin Buffer 3 for 5 min at room temperature.

16. Incubated in colour substrate solution (26.4 ul nitroblue tetrazolium; 20 µl bromochloroindolyl phosphate; 8 µl levamisole (Sigma); 5 ml Digoxigenin Buffer 3) at room temperature overnight.

17. Washed in water.

18. Counterstained with haematoxylin and mounted in glycerol gelatin (Sigma).

ISH Hybridisation Buffer:

2 M Tris pH 7	1 mM
100X Denharts (Sigma)	1X
20X SSC	2X
Formamide (Sigma)	50% (vol/vol)
20% SDS (Sigma)	0.5%
10% dextran sulphate (Sigma)	1%
Herring sperm DNA (Sigma, 10 mg/ml)	0.25 mg/ml

Digoxigenin Buffer 1:

80 mM Tris HCl, 20 mM Tris base and 150 mM NaCl

Digoxigenin Buffer 3:

As for SB Buffer 3

APPENDIX E

Pathogenicity Studies: Methods and Reagents

Bouin's Fluid

Saturated picric acid solution	75 ml
40% formaldehyde	25 ml
Glacial acetic acid	5 ml

Coomassie Blue

1% brilliant blue G (Sigma) in destain solution, prepared from 5% acetic acid, 50% methanol and 45% water.

Glutaraldehyde Fixative

Glutaraldehyde fixative was prepared by mixing 33 ml sodium cacodylate buffer and 12 ml 25% glutaraldehyde (TAAB Laboratories Equipment Ltd), adjusting the pH to 7.2 with 0.2 M HCl , and making up to 100 ml with distilled water.

Sodium Cacodylate Buffer

Sodium cacodylate buffer was prepared by adding 1g of sucrose to 33 ml sodium cacodylate stock solution, adjusting the pH to 7.2 with 0.2 M HCl and making up to 100 ml with distilled water. The sodium cacodylate stock solution was prepared from 43 g of sodium cacodylate (Agar Scientific Ltd) in 1 L distilled water.

Oncor Apoptaq™ ISH Apoptosis Detection Kit Protocol

Coverslips of cells exposed to amoebae in the transwell apparatus were treated as follows:

1. Quenched with 3% H_2O_2 for 5-10 min.

2. Washed with PBS.

5. Incubated in equilibration buffer for up to 30 min.

6. Incubated with TdT enzyme (diluted 1:3 in reaction buffer) at 37 ^{0}C for 60 min.

7. Incubated with agitation in prewarmed stop/wash buffer (diluted 1 ml in 34 ml distilled water) at 37 ^{0}C for 30 min.

8. Washed with PBS.

9. Incubated with anti-digoxigen-peroxidase conjugate (diluted 49 µl in 56 µl of blocking solution) in a humidified chamber at room temperature for 30 min.

10. Washed with PBS.

11. Visualised with DAB.

longed medical treatment is necessary before complete resolution is achieved. Early diagnosis is prerequisite[6] if this is to be achieved by medical treatment alone. However, this may be confounded by the similarity in appearance of the disease to that of herpetic or occasionally adenovirus keratitis and to the relative rarity of *Acanthamoeba* keratitis.[7]

There are reports both in the literature[8] and anecdotally of presumed *Acanthamoeba* keratitis where treatment with drugs recommended for this infection resulted in a medical cure. In these cases, amebae were not isolated using culture or were not detected by histologic examination of corneal tissue. In these circumstances, clinical behavior and therapeutic response to anti-amebic drugs were considered adequate evidence on which to base the diagnosis of *Acanthamoeba* keratitis. We present a case of amebic keratitis in a wearer of disposable soft contact lenses where *Acanthamoeba* could not be detected in corneal tissue using conventional culture techniques. Prolonged culture of stromal tissue in a defined medium yielded viable free-living amebae of the genera *Vahlkampfia* and *Hartmannella*, but no *Acanthamoeba*. These findings were supported by transmission electron microscopy of corneal stromal elements. Implications for diagnosis and treatment of this dual amebal infection are discussed.

Case Report

A 21-year-old male surveyor (GM) had been wearing disposable contact lenses (Food and Drug Administration group 4) for 18 months to correct low myopia (right eye, −2.25/−0.25 × 100 and left eye −3.75/−0.25 × 110). The lenses were replaced every 2 or 3 weeks. The lenses were removed at night and placed in a storage case that was regularly rinsed in domestic tap water. A chlorine-based disinfection system was used with tap water as diluent.

In December 1991, the left eye became painful and inflamed. This was diagnosed initially as a herpetic disciform keratitis. Treatment was with betamethasone drops (4 times daily) and acyclovir ointment (5 times daily), but there was no discernible improvement in signs or symptoms. The condition followed a remitting and relapsing course for 4 months. At this point, the patient was referred to one of us (CMK) for further management.

The patient reported loss of vision, marked ocular pain, photophobia, and a profuse watery discharge from the left eye. The right eye was essentially normal with a corrected visual acuity of 20/15. Acuity of the left eye was hand motions. The cornea presented a small (3 × 1 mm) epithelial defect overlying a circular area of corneal edema of approximately 5 mm in diameter. Underlying folds of Descemet membrane were evident as were large keratic precipitates on the underlying endothelium. There was slight flare and cells were occasionally noted in the anterior chamber. The pupil was semidilated; there were no posterior synechiae. The intraocular pressure was normal at 16 mmHg.

Clinically, these features were reminiscent of a disciform keratitis due to herpes simplex but with the unusual feature of the epithelial defect. In view of the history of disposable contact lens wear, however, the possibility of a low-grade microbial infection was considered as was *Acanthamoeba*-associated keratitis.

Corneal scrapes were taken for microscopy and culture in the routine diagnostic pathologic laboratory. Two smears were made on glass slides and stained using the Gram and Giemsa techniques. Scrape material also was directly inoculated on to blood, chocolate, Sabouraud agar, thioglycolate medium, Lowenstein–Jensen medium, and into brain–heart infusion broth. Robertson cooked meat broth also was inoculated. Non-nutrient agar (1%) seeded with heat-killed *Escherichia coli* also was used to culture for *Acanthamoeba*. Tear samples and corneal scrapes were taken for viral culture.

Microscopy showed only a few inflammatory and epithelial cells; no micro-organisms were detected. Cultures for bacteria, viruses, and fungi were persistently negative. *Acanthamoeba* was not detected despite prolonged incubation at 37 °C.

Medical treatment was started with the presumptive diagnosis of a herpetic disciform keratitis with trifluorothymidine drops 2 hourly for 2 days, then five times daily simultaneously with dexamethasone drops 0.1% four times daily and atropine drops 1% twice daily. One week later, there was subjective improvement in signs and symptoms with 20/100 visual acuity. The epithelial defect had healed, but the stromal edema and folds in Descemet membrane persisted. The anterior chamber was quiet and the keratic precipitates had resolved. Two weeks after starting treatment, the patient reported increasing photophobia and deterioration in visual acuity to hand motions. Clinical examination showed that the edematous area had increased in diameter, to approximately 7 mm, and there was a plaque of confluent keratic precipitates (5–6 mm in diameter) on the endothelium. The patient was immediately admitted to hospital for observation and more intensive therapy. Prednisolone 1% drops were administered hourly; this produced resolution of the precipitates and some resolution of the stromal edema. The improvement, however, was slow over succeeding weeks.

By July 1992, the lesion had progressed. There was a central 8-mm area of edema with obvious infiltrate and again a plaque of keratic precipitate; no hypopyon was observed. Fine vessels invaded the deep stroma peripherally. Although at this stage there was some doubt as to patient compliance with drug therapy, it was considered that the original clinical diagnosis of herpetic disease was incorrect.

At this stage, *Acanthamoeba* keratitis was considered more likely. Drug treatment was withdrawn and, 48 hours later, a corneal biopsy was performed (Fig 1A). The epithelium was removed in the superior quadrant and fixed in cacodylate-buffered glutaraldehyde for subsequent electron microscope examination. Two separate biopsies were taken using a 3-mm trephine. Selected biopsy sites spanned the edge of the abscess. The trephine marks were deepened to approximately 90% corneal depth using a razor-blade fragment. A lamellar dissection completed the biopsy so that the specimen included tissue deep to the stromal abscess. One specimen was forwarded for microbiologic examination and the other for histopathologic examination. Initial morphology identified amebae in the epithelium and stromal specimens, although the genus of the protozoa could not be determined. Treatment was started with 0.1% propamidine Brolene isethionate drops hourly and neomycin 0.5% hourly for 2 weeks; this was then reduced to 2 hourly (subsequently 3, then 4 hourly) with 0.15% dibromopropamidine Brolene isethionate ointment at night.[9] This was the standard treatment, in use in 1992, in our department for *Acanthamoeba* keratitis. Once culture of "vahlkampfia-like" ameba had been made, the patient was treated with Amphotericin B drops (intolerance to this drug required its rapid withdrawal). Atropine 1% drops once a day were administered with prednisolone drops 1% in slowly reducing course.

Clinical improvement was rapidly apparent using this treatment regimen. There was then a more gradual decrease in the size of the abscess over the course of 8 months. A central corneal scar remained with some vascularization and an epithelial defect

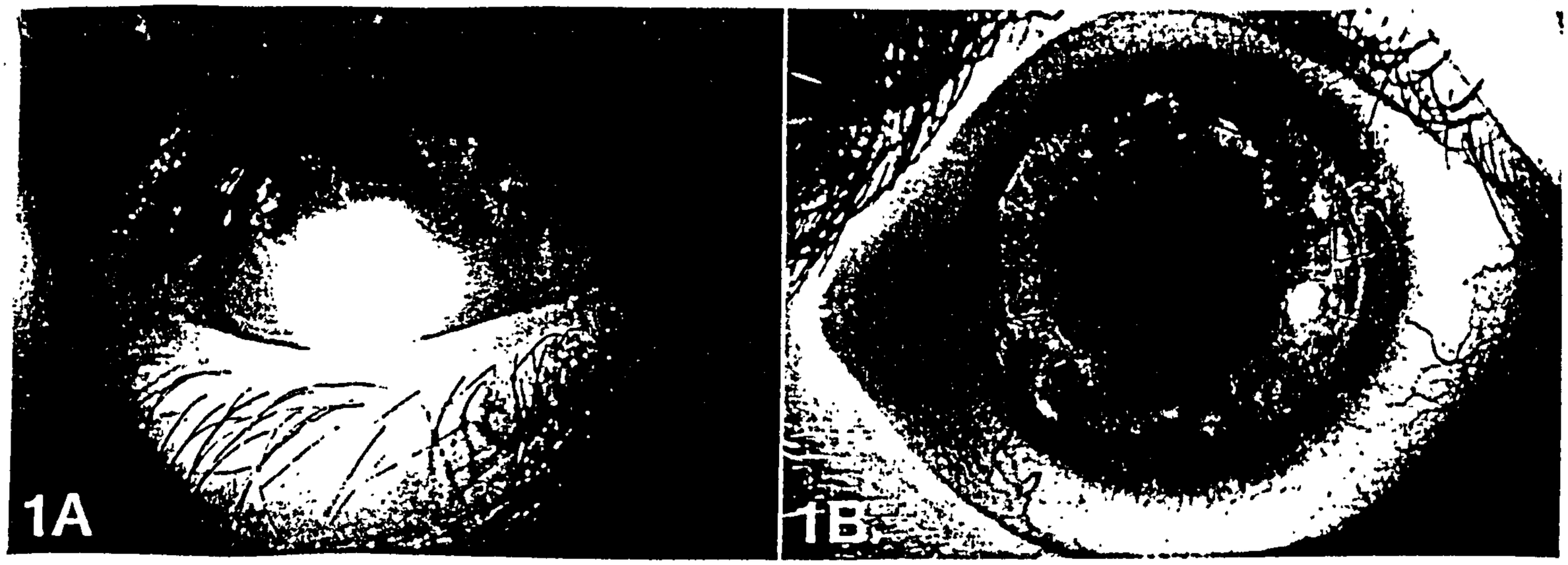


Figure 1. Clinical appearance of the ulcer. A, the appearance of the eye after biopsy and beginning of treatment. The two biopsy sites can be seen :riorly at the edge of the abscess. B, appearance of the cornea 1 year after transplantation.

of approximately 3 mm in diameter. The eye was quiet and comfortable on treatment with propamidine drops isethionate. neomycin sulfate 0.5%. and prednisolone 1%. all four times daily and atropine drops once per day.

One year after starting drug therapy. the eye was stable with no discernible signs of inflammation. There was central corneal scarring: fine corneal blood vessels also were evident. Visual acuity was 1/200. There was no afferent pupillary defect. The ..ocular pressure was normal. An 8-mm penetrating kera-..plasty was performed with no complications. Postoperatively. prednisolone forte eyedrops were administered 2 hourly with cyclopentolate once per day: propamidine and neomycin were continued 4 hourly. The eye remained quiet. There was no inflammation and no discernible evidence of infection. Steroid therapy was reduced to four times daily after 4 weeks. After 8 weeks. neomycin was withdrawn. After 4 months. visual acuity was 20/30 with −3.5 diopters. Twenty-one months later. the graft remains clear with a normal intraocular pressure (Fig 1B). stable refraction. and visual acuity at 24 months postkeratoplasty.

Morphologic Results

Corneal Biopsy

Corneal epithelium subjected to paraffin histology showed non-specific secondary inflammatory changes: no microorganisms were identified in this specimen. Light microscopy on toluidine blue stained araldite sections did ..t show convincing evidence of the presence of protozoa ... other microbial pathogens. The stroma contained erythrocytes. polymorphonuclear leukocytes. lymphocytes. and macrophages. Atypical mononuclear cells that were elongated. stellate. and larger than endogenous macrophages were present in the deep stroma. Some of these atypical cells contained small inclusion bodies and vacuoles. Residual keratocytes were identified between the lamellae.

Transmission electron microscopy of the epithelium ..n the biopsy also failed to show the presence of pathogens. ..he stroma contained a similar inflammatory infiltrate to that seen by light microscopy but. in addition. cells containing digestive and contractile vacuoles were seen. These cells were strongly reminiscent of amebae (Figs 2A and 2B) and were not typical of *Acanthamoeba*[10] but were similar to *Vahlkampfia* and *Hartmannella*.[11,12] A characteristic feature of both polymorphonuclear leukocytes and amebae was their restriction to interlamellar space (Figs 2A–2C). In addition. the amebae possessed a striking electron dense cytoplasm. bounded by an undulating plasmalemma with focal densifications (Figs 2B and 2C). Elongated trophozoites containing vacuoles were observed (Fig 2D). Nuclear detail necessary for unequivocal identification of the genus was not apparent. principally due to the plane of sectioning.

Keratoplasty Disc

Paraffin histology and transmission electron microscopy were performed on half of a corneal button. Hematoxylin and eosin stained sections showed that the stroma contained scattered blood vessels and mononuclear cells. Bowman layer and Descemet membrane were duplicated with some splitting. The endothelium was absent.

Initial immunohistochemistry performed on these sections using a rabbit polyclonal anti-*Acanthamoeba* antiserum (donated by Dr. D. Warhurst) and peroxidase–antiperoxidase labeling showed presence of circular structures and debris. which stained positively in the posterior stroma (Fig 3A). Further immunofluorescent studies were undertaken (refer to those below and to Fig 3B).

A plastic-embedded section stained with toluidine blue showed a hyperplastic epithelium: there was a compact stroma and a cellular infiltrate. This was confirmed by electron microscopy where collapsed cysts. either empty or enclosing cytoplasmic debris. were found between the lamellae. These structures were surrounded by debris comprising vesicles. granular material. and electron dense bodies (Figs 4A and 4B).

Contact Lens, Storage Case, and Case Fluid

Scanning electron microscopy (SEM) showed that the surface of the contact lens was lined by scattered bacteria.

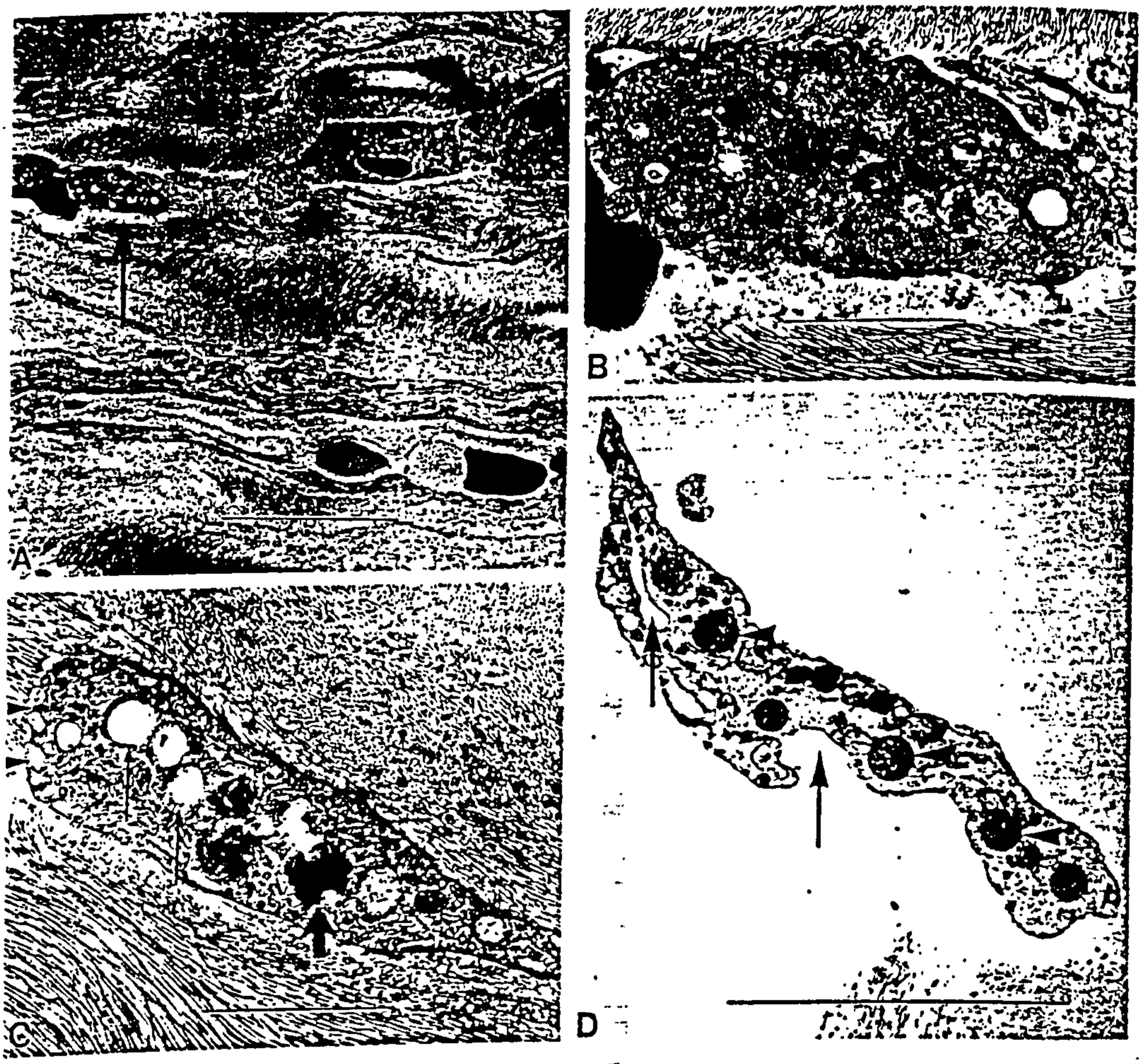


Figure 2. A, in the lamellar corneal biopsy, an atypical cell presumed to be a trophozoite (arrow). Polymorphonuclear leukocytes and cells with atypical inclusion bodies were present between the corneal lamellae (transmission electron microscopy, bar = 10 μm). B, the cytoplasm of these atypical cells (in 2A) contains vacuoles characteristic of free-living amoeba. C, features that indicate the presence of a trophozoite include apical expulsion vacuoles (arrowhead) and lipid vacuoles (fine arrows) and electron-dense nuclear chromatin (solid arrow) (triethylenemelamine, bar = 2 μm). D, this organism has features characteristic of a limax trophozoite, with tapering end, expulsion vacuoles (arrows), and food or digestive vacuoles (arrowheads) (transmission electron microscopy, bar = 5 μm).

yeast-like fungal elements and a variety of protozoa in different stages of development. These protozoa had cytoplasmic extrusions that projected into surface defects of the contact lens (Figs 5A and 5B). The flocculent suspension in the lens case fluid was fixed in cacodylate-buffered glutaraldehyde (2%) and centrifuged into a loose

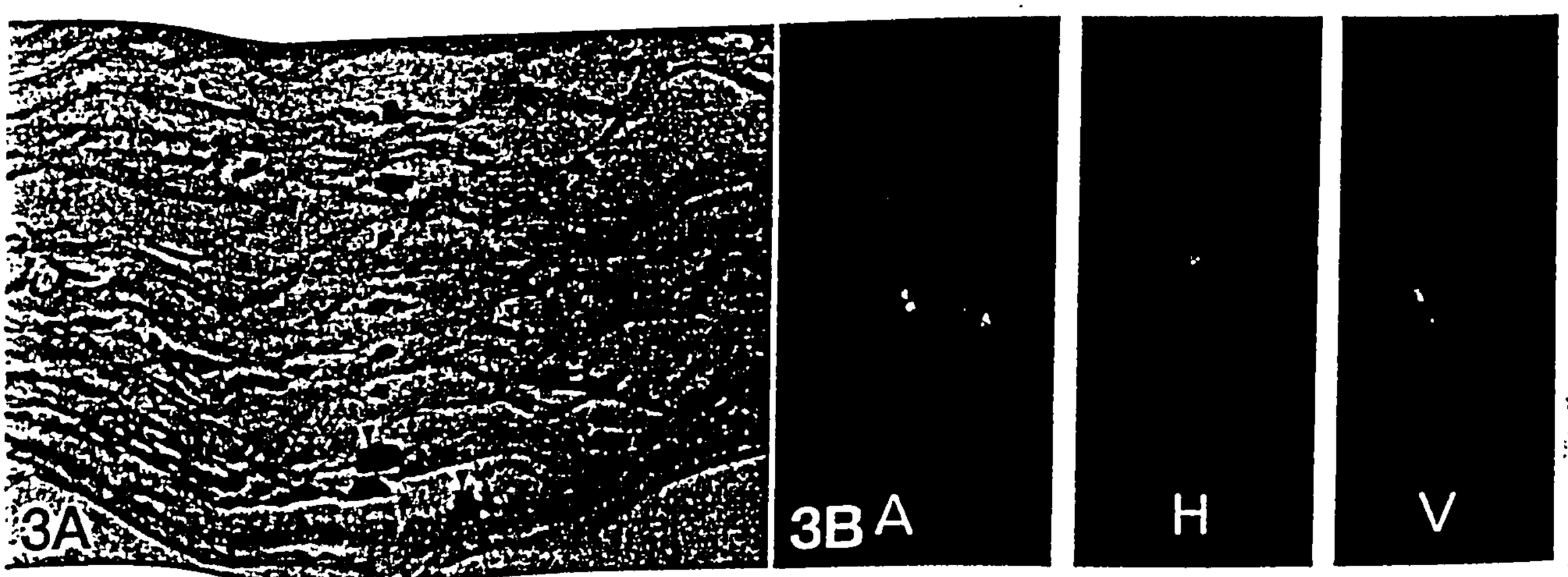


Figure 3. A, in the posterior stroma of the keratoplasty disc, oval structures (arrowheads) and granular material stained positively with anti-acanthamoebal antisera (1:640 dilution) (peroxidase-antiperoxidase stain; original magnification, ×330). B, labeling of *Acanthamoeba*, (A), *Hartmannella*, (H), and *Vahlkampfia*, (V), with anti-*Acanthamoeba* Neff polyclonal antiserum (1:150 dilution as primary layer in each case). Cross-reactivity is apparent.

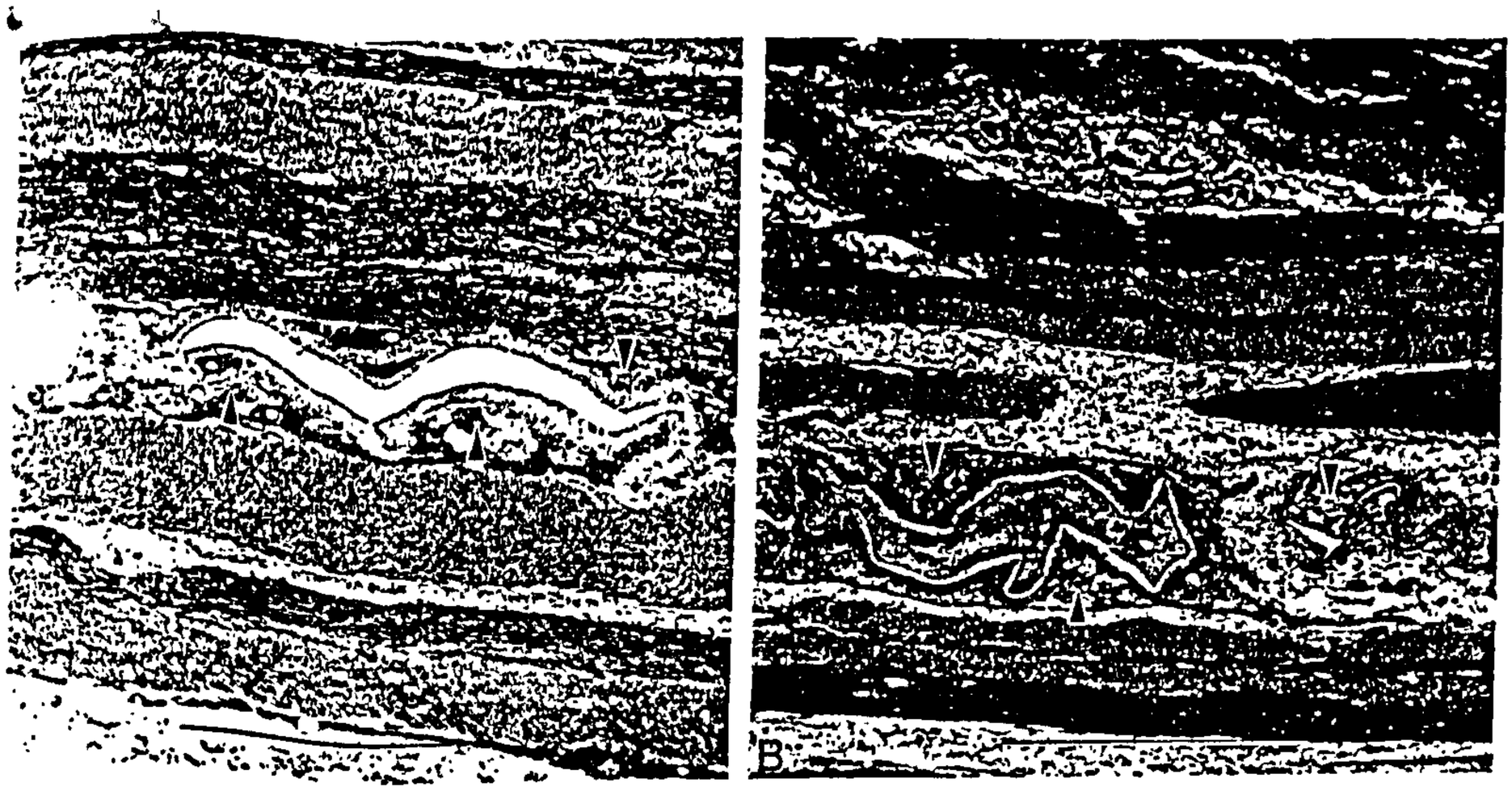


Figure 4. Collapsed cyst walls surrounded by cytoplasmic debris (arrowheads). Features are morphologically dissimilar to degenerate keratocytes (triethylenemelamine, bar = 5 μm).

pellet. The pellet was processed for transmission electron microscopy but divided at the absolute alcohol stage to provide material for SEM. The portion for SEM was resuspended in 100% alcohol, spread on to aluminum foil, dried in air, and gold coated. Various, different protozoa were identified on a proteinaceous matrix that contained a diverse range of other smaller micro-organisms (Fig 6A). Comparison of these organisms with similarly prepared pure axenic cultures showed the presence of *Acanthamoeba*, *Hartmannella*, and *Vahlkampfia* trophozoites and cysts. Other less recognizable protozoa, a variety of fungi, and filamentous algae as well as a morphologically diverse range of bacteria also were identified (Fig 6B).

Home Water Supply

Scanning electron microscopy of a biofilm sample taken from the bathroom cold water supply faucet rim also yielded *Acanthamoeba*, *Hartmannella*, and *Vahlkampfia* as well as flagellated protozoa, desmids, and diatoms (Fig 6B). Various bacteria also were identified. Other organisms present included filamentous algae and fungi.

Microbiologic Studies

Biopsy and Keratoplasty Specimens

Tissue from the corneal biopsy was dissected using a sterile scalpel blade. For bacteriologic and fungal culture, one portion was homogenized in a Griffith tube in a minimum volume of sterile isotonic saline. Aliquots (0.05 ml) were plated on to Columbia blood agar (for aerobes and anaerobes), chocolate agar, and Sabouraud medium; others were instilled into Robertson cooked meat broth and brain–heart infusion broth (with and without salt). The remainder was used for protozoologic examination. Tissue was teased apart using sterile needles. Smears were made for direct wet mount microscopy. Aliquots of a homogenate as well as small tissue samples were plated on to 1.5% or 0.5% high-clarity bacteriologic agar No. 1 (LABM) made up in Page amebal saline.[13] Heat-killed Klebsiella aerogenes was included as an amebal nutrient source. Plates were moistened intermittently with amebal saline while incubating in air at 25°C, 32°C, or 35°C. No pathogenic bacteria or fungi were detected in any of the cultures.

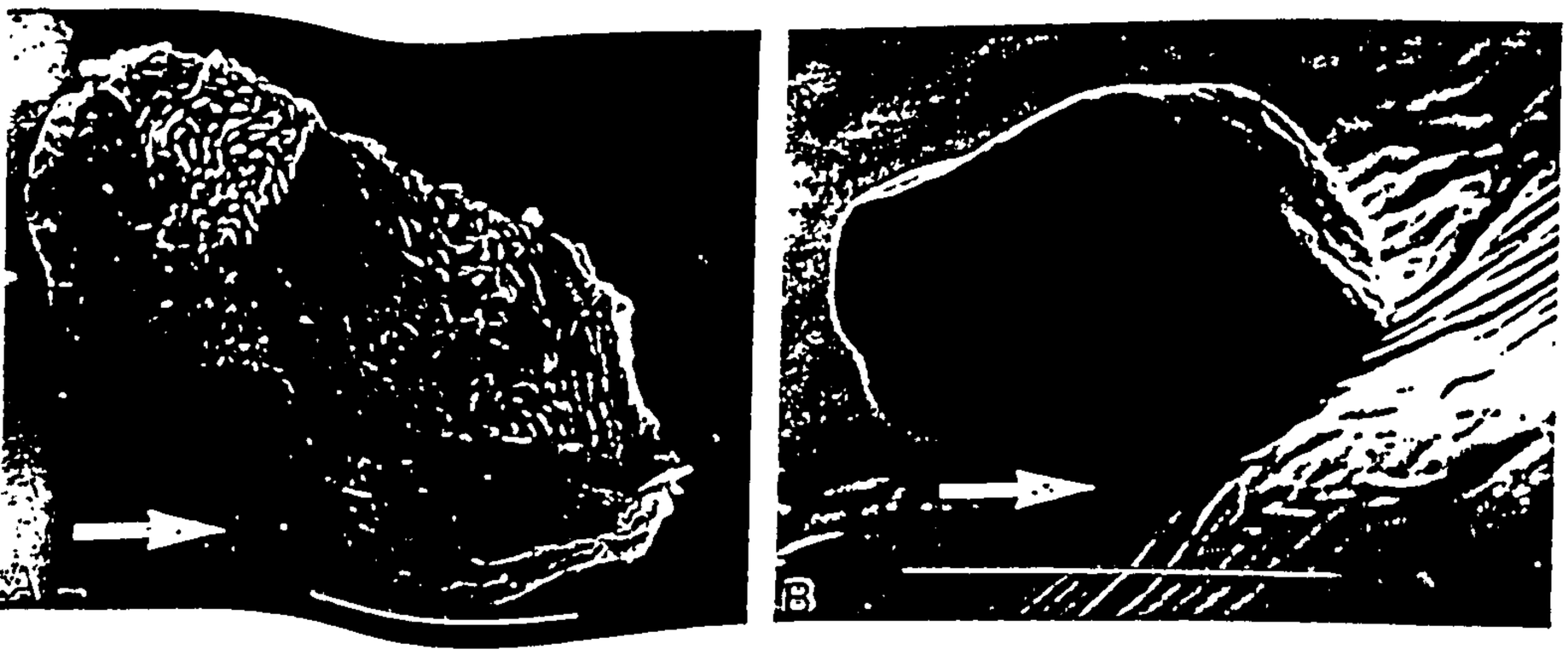


Figure 5. A, trophozoite with ruffled surface and cytoplasmic extension protruding (arrow) into a pit on the contact lens surface (scanning electron microscopy, bar = 10 μm). B, sessile amoebal form anchored (arrow) to the contact lens surface (scanning electron microscopy, bar = 10 μm).

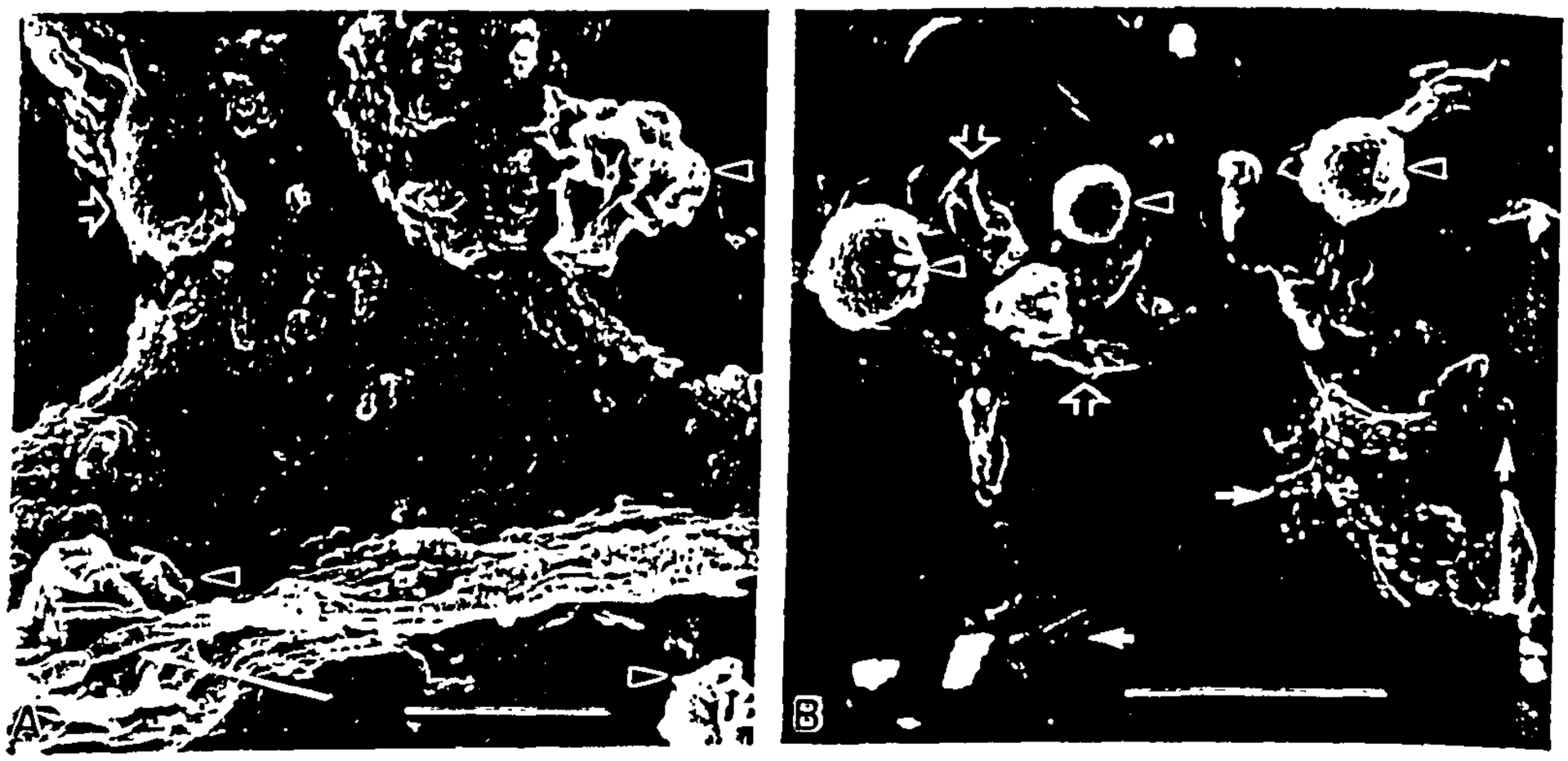


Figure 6. A, the organisms obtained from the contact lens fluid compromised readily identifiable amoebal trophozoites (open arrow) and cysts (arrowheads) as well as fungal elements (white arrow) on a matrix containing bacteria (scanning electron microscopy, bar = 10 μm). B, biofilm of cold water faucet rim with spherical bodies, *Hartmannella* cysts (arrowheads), and bacteria (white arrowheads) and flagellates (open arrows) (scanning electron microscopy, bar = 10 μm).

There was no perceptible growth from the samples using this approach, although two cyst-like structures were tentatively identified after 5 days incubation in the 0.5% agar incubated at 32°C. These did not correspond to the usual morphologic appearance of *Acanthamoeba* and were not readily reminiscent of other amebal cysts and were initially considered to be cellular debris from corneal tissue.

After 14 days, aliquots of the cultures were transferred into sterile glass Bijoux bottles containing approximately 0.5 ml of a defined medium[14] and cultivated in this medium at 32°C. Approximately 3 days later, a sparse growth of vahlkampfia-like trophozoites was noted in the samples. After several passages that permitted adaptation of the amebae to the culture medium, these protozoa were transferred to 1.5% non-nutrient agar, where confluent growth of *Vahlkampfia* and *Hartmannella* ensued (Fig 7). No *Acanthamoeba* was identified. The penetrating keratoplasty specimen was treated in a similar manner as the biopsy specimen. No amebae or bacterium was detected even after prolonged incubation.

Contact Lens Storage Case and Water Samples from the Home

Fluid from the clear plastic storage case was decanted into a sterile glass universal container. A thin, gray–green biofilm remained. This biofilm was removed using a sterile sponge swab. The contact lens was vigorously agitated in sterile isotonic saline and aliquots subjected to the procedures outlined above for microbiologic examination of the biopsy and keratoplasty samples.

Water samples (5 l) were collected from the mains-supplied kitchen cold water faucet as well as the tank-supplied bathroom hot and cold water faucets. The latter is where the patient performed his contact lens hygiene practice. Agitated water from the loft storage tank and residue on the ballcock, biofilm on the tank overflow, and from the interior of the bathroom faucets and hand basin overflow were collected. All water samples and the storage case fluid were filtered through individual 0.45-μm filters (Gelman Services). The filter was then shredded and processed on to agar in a similar manner as for the ocular samples. Swabs were placed in approximately 0.5 ml of amebal saline[13] contained within a glass centrifuge tube and mixed using a vortex mixture. The resulting fluid was then treated in a similar manner as for the ocular samples. Results of microbiologic examination of these samples are listed in Table 1.

Drug Screening

Once the two genera were separated,[15,16] they were transferred to individual non-nutrient agar plates and cultured for subsequent drug sensitivity testing.[17] Plates containing trophozoites, cysts, or both were flooded with amebal saline using a Pastette disposable pipette and agitated to permit transfer of amebae to sterile plastic 75 cm^2 tissue culture flasks (Sterilin, Celcult, Southampton, UK) con-

Figure 7. Cluster of *Vahlkampfia* cysts and a single *Hartmannella* cyst (arrow) from a culture. Note distinct gelatinous coating on smooth wall of the *Vahlkampfia* (original magnification, ×3700).

taining approximately 50 ml of the axenic medium described above. After several transfers that acted to habituate the amebae to the medium, flasks were incubated at 32°C, the temperature of their optimum growth. Drugs used were chlorhexidine digluconate, polyhexamethylene-guanide, propamidine Brolene isethionate, pentamidine isethionate, neomycin sulfate, paromomycin sulfate, and amphotericin B. Combination antimicrobial testing was performed as described previously.[17] Results of this testing are listed in Table 2.

Immunocytochemical Staining

To verify the genera of free-living amebae infecting the cornea, the sensitivity and specificity of a range of polyclonal antisera against *Acanthamoeba*, *Hartmannella*, and *Vahlkampfia* were investigated.

Cultures of each protozoan genera were obtained from the Culture Collection of Algae and Protozoa. Individual polyclonal antisera were prepared by inoculation into a rabbit of a mixed suspension of formaldehyde solution-killed trophozoites and cysts (10^5 per ml) in incomplete Freund adjuvant, followed 24 days later by inoculation of live protozoa (10^5) in the absence of adjuvant. Four weeks later, the animal was exsanguinated. In addition, another two preparations of antisera raised in rabbits against *Acanthamoeba* were made available by Dr. D. Warhurst. Immunocytochemical staining was performed on smears of axenically cultured strains of *Acanthamoeba*, *Hartmannella*, and *Vahlkampfia* as follows:

1. Dried smears containing amebae were fixed in acetone for 2 minutes.
2. Smears were washed thoroughly in TRIS-buffered saline (TBS), pH 7.6.
3. Nonspecific reactions were blocked using 1:5 normal swine serum in TBS (DAKO, Copenhagen, Denmark).
4. Smears were washed in TBS and excess buffer was removed.
5. The primary layer comprising the particular antibody under test (1:25 normal swine serum in TBS) was added for 30 minutes.

Table 1. Culture Results

Tissue Samples	
Corneal scrape	No pathogenic bacteria or viruses detected
Corneal biopsy	No pathogenic bacteria or viruses detected *Vahlkampfia* detected *Hartmannella* detected
Penetrating keratoplasty	No microorganisms isolated
Environmental Samples	
Water	
Kitchen cold water faucet	No amoebae isolated
Kitchen hot water faucet	No amoebae isolated; diatoms
Bathroom cold water faucet	No amoebae isolated; diatoms
Bathroom hot water faucet	No amoebae isolated; diatoms
Roof storage tanks	Ciliates, algae, diatoms, desmids; no amoebae isolated
Swabs of biofilm	
Ballcock of storage tank	Algae, diatoms, desmids
Tank overflow	Algae, diatoms, desmids
Kitchen faucets	Ciliates, flagellates, algae
Bathroom faucets	*Vahlkampfia*, *Hartmannella*, *Acanthamoeba*, *Vexillifera*, peritrichs, other ciliates, filamentous algae; unidentified free-living nematodes
	Mixed bacterial growth; mixed coliforms and yeasts
Contact lens storage case	
Fluid	*Vahlkampfia*, *Hartmannella*, *Acanthamoeba*, *Vexillifera*, *Bodo saltans*, and other peritrichs, ciliates, filamentous algae; unidentified free-living nematodes
Biofilm	Amoebae, flagellates, and ciliates as in fluid

Table 2. Drug Sensitivities (minimimal amoebicidal concentrations μg/ml)

Sensitivities*	Vahlkampfia Trophozoites	Vahlkampfia Cysts	Hartmannella Trophozoites	Hartmannella Cysts
Chlorhexidine	3.2	12.5	6.3	12.5
Polyhexamethyline biguanide	3.2	6.3	3.2	12.5
Neomycin	6.3	50.0	6.3	25.0
Paromomycin	12.5	50.0	25.0	50.0
Propamidine	3.2	12.5	6.3	12.5
Pentamidine	6.3	25.0	12.5	50.0
Amphotericin B	1.6	12.5	3.2	25.0

* Combinations of chlorhexidine + propamidine – additive; neomycin + propamidine – autonomous; chlorhexidine + amphotericin B – autonomous

6. Smears were washed with agitation in TBS for two periods of 5 minutes each and excess buffer was removed.
7. The secondary layer comprising fluorescein-isothiocyanate, labeled swine anti-rabbit immunoglobulins (1:20 in 1:25 normal swine serum in TBS), was added for 30 minutes.
8. Step 6 was repeated.
9. The preparation was mounted (Univert mountant; Searle Scientific Services. High Wycombe) and examined by ultraviolet microscopy.

Control specimens comprised amebal preparations in which the primary layer, secondary layer, or both had been omitted. Individual slides were coded and independently assessed by a series of observers. Untreated amebal cysts had inherent immunofluorescence, which was found to dissipate after acetone fixation. A scoring system was devised as follows: (1) 0, not labeled: (2) +, labeled but not diagnostic; and (3) ++, diagnostic labeling.

The optimum titers for each antibody preparation are listed in Table 3. Figure 3B demonstrates labeling of *Acanthamoeba, Vahlkampfia.* and *Hartmannella* with an anti-*Acanthamoeba* antisera. demonstrating cross-reactivity. It is our experience that such antibodies may also label epithelium. and to a certain extent other tissues, in ameba-infected corneal specimens (Fig 3A).

Discussion

Certain free-living phagotrophic amebae are opportunistic pathogens of humans.[18] *Naegleria fowleri* is responsible for rapidly fatal primary amebic encephalitis. Several species of *Acanthamoeba* can cause a granulomatous meningo-encephalitis and the well-recognized keratitis. The Leptomyxida ameba *Balamuthia mandrillaris* has been shown to cause meningo-encephalitis in humans.[19] A fatal case of meningo-encephalitis also has been reported in association with *Hartmannella vermiform.*[20] Two cases of encephalitis. probably due to *Vahlkampfia.* have been reported, the diagnosis being based on the morphologic appearance of the amebae in tissue sections.[21]

It might appear that *Hartmannella* has been the cause of keratitis and other ocular diseases.[22] This is the consequence of a previous but now resolved debate on the taxonomic status of *Acanthamoeba.* where *Acanthamoeba* and *Hartmannella* often were classified as the single genus, *Hartmannella.* The two genera are now considered tax-

Table 3. Immunofluorescence—Optimal Endpoint Titers of Polyclonal Antiamoebal Sera Used in this Study

Isolate	Rabbit Immunized Against				
	*Acanthamoeba Neff**	*Acanthamoeba castellanii**	*Acanthamoeba castellanii*	*Vahlkampfia avara*	*Hartmannella vermiformis*
Acanthamoeba†	1:30	1:30	1:15	1:5	1:15
Acanthamoeba‡	1:30	1:30	1:15	1:5	1:15
Vahlkampfia§	1:30	1:30	1:15	1:5	1:15
Hartmannella§	1:30	1:30	1:15	1:5	1:15

* Donated by Dr. D. Warhurst.
† *Acanthamoeba* from lens storage case (GM)
‡ *Acanthamoeba* isolate from the cornea of patient (AB).[17]
§ Isolates from (GM) cornea.

onomically distinct.[23] *Hartmannella* and *Vahlkampfia* are both free-living amebae and may be found in water and soil, and some species are causal agents of disease in invertebrates and fish. *Vahlkampfia* is in the same family (vahlkampfidae) as is Naegleria, and the trophozoites of both amebae are markedly eruptive in their mobility.

Hartmannella trophozoites are monopodial except when changing direction. The cysts are small and have a characteristic morphology.[13] These characteristics may be used as part of the identification process.

Until now, no other free-living amebae apart from *Acanthamoeba* has been recorded to cause keratitis in humans or animals. In the case presented here, *Hartmannella, Vahlkampfia,* and *Acanthamoeba* were present in association with keratitis. These protozoa were cultured from corneal tissue and were observed ultrastructurally within the stroma at biopsy. *Hartmannella, Vahlkampfia,* and *Acanthamoeba* were detected in the contact lens storage case and were isolated from the domestic water supply in the patient's home. This suggests the possible route of infection from the biofilm on the faucets through the water to the contact lens case and thus by direct transfer to the ocular surface. The heavy microbial load present in the case and on the contact lens would have facilitated corneal insult to the cornea, permitting opportunistic invasion of the cornea by the amebae.[24] *Acanthamoeba* could not be cultured from the cornea nor could it be demonstrated unequivocally by light or transmission electron microscopy. Clinically, the infection followed a prolonged fluctuating course and was poorly responsive to topical steroids and antiviral drugs, initially having been mistaken for herpetic kerato-uveitis. At presentation, no corneal abscess was present. Stromal edema was present with increasing inflammation of the endothelium. Pain was not a prominent feature until late in the course of infection. This is in contradistinction to the usual features of an *Acanthamoeba*-associated keratitis where radial keratoneuritis is usually associated with intolerable pain at a relatively early stage.[7]

The natural progression of the amebic infection may have been attenuated by the various topical treatments administered, although acyclovir does not inhibit the growth of *Acanthamoeba* in vitro (unpublished data, Tennent Institute, 1994). Nevertheless, the disease process was slower than that generally observed with *Acanthamoeba*, taking almost 7 months before formation of a corneal abscess. The initial response to treatment was quicker than is usual in a case of advanced *Acanthamoeba* keratitis. Soon after starting antimicrobial chemotherapy, the eye became comfortable and the inflammation subsided gradually. The overall course of the infection remained protracted, however, with healing of the epithelial defect taking approximately 9 months of medical treatment. Although it was some 7 months before definitive diagnosis of an amebal infection was made, the infiltrate remained confined to the central corneal stroma, which had not thinned significantly, and thus there was no likelihood of scleral invasion. These clinical observations present a distinctly different pattern of behavior from what has become widely recognized as typical for an *Acanthamoeba*-associated amebal keratitis.

The clinical progression in the present expanded case report was sufficiently similar in some respects for *Acanthamoeba* infection to have been incorrectly accepted as the diagnosis. In particular, there was considerable circumstantial evidence provided by a favorable response of the infection to the combination therapy of propamidine and neomycin, which has until now provided satisfactory resolution in some cases of culture and histologically proven *Acanthamoeba* keratitis. The potential efficacy of amphotericin B, shown to be more effective against both the *Vahlkampfia* and *Hartmannella* than *Acanthamoeba* in vitro, could not be tested in vivo because of patient intolerance to this drug. Drug sensitivity studies should be performed on the corneal isolates because there appears to be a correlation between in vitro sensitivity to antiprotozool agents and associated in vivo clinical response; this has been shown with a range of drugs against different *Acanthamoeba* strains.[17]

It is likely that in some instances, ameba other than *Acanthamoeba* may be involved in cases of presumed amebic keratitis. These cases may be reported as presumed *Acanthamoeba* keratitis or as cases in which the *Acanthamoeba* failed to be cultured. There is no evidence from these reports to substantiate the unequivocal diagnosis of *Acanthamoeba* keratitis. In some cases of culture-negative *Acanthamoeba* keratitis, ameba have been seen in tissue by microscopy or have been seen by immunohistochemical staining. We suggest that these techniques may not be conclusive in distinguishing genera because nonadsorbed anti-*Acanthamoeba* antisera also may cross-react with *Hartmannella* and *Vahlkampfia*, suggesting shared antigenic epitopes. Furthermore, *Hartmannella* and *Vahlkampfia* were more difficult to culture than is usual for *Acanthamoeba*. This may explain why cases with amebae, seen in tissue, remain culture-negative.

We suggest that in future investigations of presumed amebic keratitis, where *Acanthamoeba* cannot readily be cultured or identified using morphology or polymerase chain reaction and hybridization sequences[25] when available, other free-living amebae should be considered in the diagnosis. Immunofluorescent polyclonal antisera may be helpful for detecting the presence of amebae. Viability should be investigated using enriched axenic media incubated at 25°C and 32°C.

It is our belief that the findings of this case are not unique. Like *Acanthamoeba*, the free-living amebae *Hartmannella* and *Vahlkampfia* are ubiquitous and cosmopolitan in the environment[26] where they inhabit soil, fresh water, including domestic supplies and marine water, and air. Therefore, we confidently predict that other cases of *Hartmannella* and *Vahlkampfia* keratitis will be identified.

Addendum

Since the submission of this article, a report has been published of keratitis associated with *Hartmannella ver-*

miform in a 17-year-old wearer of soft contact lenses.[27] There was a stromal infiltrate typical of *Acanthamoeba* infection. The patient was treated with 0.02% (sic 0.2%) polyhexamethylene-biguanide.

References

1. Moore MB. McCulley JP, Newton C, et al. Acanthamoeba keratitis. A growing problem in soft and hard contact lens wearers. Ophthalmology 1987;94:1654–61.
2. Sharma S. Srinivasan M, George C. Acanthamoeba keratitis in non-contact lens wearers. Arch Ophthalmol 1990:108: 676–8.
3. Seal DV. Hay J. Munro FA. Acanthamoeba keratitis: a waterborne disease. British Journal of Optometry and Dispensing 1994;2:475–9.
4. Moore MB. Acanthamoeba keratitis and contact lens wear: the patient is at fault. Cornea 1990:9(Suppl 1):S33–5.
5. Stehr-Green JK. Bailey TM. Visvesvara GS. The epidemiology of Acanthamoeba keratitis in the United States. Am J Ophthalmol 1989:107:331–6.
6. Bacon AS. Dart JK. Ficker LA. et al. Acanthamoeba keratitis. The value of early diagnosis. Ophthalmology 1993:100:1238–43.
7. Kirkness CM. Hay J. Seal DV. Aitken D. Acanthamoeba keratitis. Ophthalmol Clin North Am 1994:7:605–16.
8. Bacon AS. Frazer DG. Dart JK. et al. A review of 72 consecutive cases of Acanthamoeba keratitis 1984–1992. Eye 1993:7:719–25.
9. Wright P. Warhurst D. Jones BJ. Acanthamoeba keratitis successfully treated medically. Br J Ophthalmol 1985:69: 778–82.
10. Pussard M. Pons R. Morphologie de la parokystique et taxonomie du genre Acanthamoeba (Protozoa. Amoebida). Protistologica 1977:13:557–98.
11. Page FC. A light and electron microscope comparison of limax and flagellate. marine amoebae belonging to four genera. Protistologica 1980:16:57–78.
12. Page FC. The limax amoebae: comparative fine structure of the Hartmannellidae (Lobosea) and further comparisons with Vahlkampfidae (Heterolobosea). Protistologica 1985: 21:361–83.
13. Page FC. A New Key to Freshwater and Soil Gymnamoebae. Ambleside Cumbria: Freshwater Biological Association, 1988:16–9.
14. Byers TJ. Akins RA. Maynard BJ. et al. Rapid growth of Acanthamoeba in defined medium: induction of encystment by glucose-acetate starvation. J Protozool 1980:27:216–9.
15. Neff RJ. Purification, axenic cultivation and description of a soil amoeba. Acanthamoeba sp. J Protozool 1957;4:176–82.
16. Neff RJ. Mechanisms of purifying amoebae by migration on agar surfaces. J Protozool 1958:5:226–31.
17. Hay J. Kirkness CM. Seal DV. Wright P. Drug resistance and Acanthamoeba keratitis: the quest for alternative antiprotozoal chemotherapy. Eye 1994:8:555–63.
18. John DT. Opportunistically pathogenic free-living amebae. In: Kreier JP. Baker JR. eds. Parasitic Protozoa. 2nd ed. Vol. 3. San Diego: , 1993: 143–246: chap 4.
19. Visvesvara GS. Schuster FL. Martinez AJ. *Balamuthia mandrillaris* N.G.. N.Sp.. agent of amebic meningoencephalitis in humans and other animals. J Eukaryot Microbiol 1993:40:504–14.
20. Rivera F. Centeno M. Cerva L et al. Fourth International Congress of Protozoology. Berlin. Germany. J Eukaryot Microbiol 1993:40:53A.
21. de Jonckheere JF. Epidemiology. In: Rondanelli EG. ed. Infectious Diseases. Color Atlas Monographs. 1. Amphizoic amoebae: Human pathology. Pavia. Italy: Piccin Nuova Libraria. 1987:127–47: chap 5.
22. Tabbara KF. Other parasitic infections. In: Tabbara KF. Hyndiuk RA. eds. Infections of the Eye. Boston: Little, Brown. 1986:679–95.
23. Martinez AJ. Free-living amebas: natural history. prevention. diagnosis. pathology. and treatment of disease. Boca Raton. FL: CRC Press. 1985:921–2.
24. Jones DB. Acanthamoeba—the ultimate opportunist? Am J Ophthalmol 1986:102:527–30.
25. Vodkin MH. Howe DK. Visvesvara GS. McLaughlin GL. Identification of Acanthamoeba at the generic and specific levels using the polymerase chain reaction. J Protozool 1992:39:378–85.
26. Rodriguez-Zaragoza S. Ecology of free-living amoebae. Crit Rev Microbiol 1994:20:225–41.
27. Kennedy SM. Devine P. Hurley C. et al. Corneal infection associated with Hartmannella vermiformis in contact lens wearer. Lancet 1995;346:637–8.