Use of cell cultures as an indicator of pathogenicity of free-living amoebae

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SUMMARY   Results comparing the time needed for the development of cytopathic effects in cell cultures with that needed to cause death in mice using inocula of Naegleria and Acanthamoeba are presented. The significance of the source and concentration of the inocula is demonstrated. The use of cell cultures as an indicator of the pathogenicity of free-living amoebae is discussed.

There is now sufficient epidemiological evidence to include the pathogenic free-living amoebae (PFLA), which are the aetiological agents of primary amoebic meningoencephalitis (PAM), in the general group of waterborne diseases. It is becoming common practice to test for the presence of these organisms as well as for the traditional waterborne pathogens (Culbertson, 1971; Carter, 1972; Chang, 1974; Craun, 1976). The case reported by Anderson and Jamieson (1972) of a young child contracting PAM from a chlorinated domestic source, the 16 cases of the disease from an indoor municipal swimming pool (Červa, 1971a), and the numerous isolations of amoebae from public swimming areas (Chang, 1971, 1974; Červa, 1971a; Červa and Huldt, 1974; Brown and Cursons, 1977) indicate the extent of this problem.

From a public health viewpoint reliable tests for the rapid identification of both the pathogenic and nonpathogenic species of the amoebae are necessary for the control of the disease. An indirect fluorescent antibody (IFAB) method was reported to meet this need (van Dijck *et al.*, 1974; de Jonckheere *et al.*, 1975). However, the finding of mouse-nonpathogenic seropositive *Naegleria fowleri* (the causative agent of PAM) (de Jonckheere *et al.*, 1975) indicates a need for further definitive and supporting tests, as outlined by Cursons and Brown (1976), to distinguish positively between pathogenic and nonpathogenic amoebic isolates. Traditionally, this has involved the use of experimental animals, particularly mice, which we have found exhibit a considerable variation in response to infection with PAM amoebae. Such variation has also been reported by Červa (1971b), Culbertson (1971),

Anderson and Jamieson (1972), Phillips (1974), Diffley *et al.* (1976), and Adams *et al.* (1976). The alternative to an *in vitro* system is the use of chick embryos or cell cultures inoculated with amoebae for testing the virulence of environmental isolates and which can be used as a prelude to animal pathogenicity tests with considerable benefit. The purpose of this paper is to compare the reports of the use of cell cultures as indicators of pathogenicity of free-living amoebae and to report on recent experiments comparing the use of VERO (secondary monkey kidney cells) with mice and/or guinea-pigs.

Material and methods

AMOEBAE

N. fowleri strain HB-1, *Naegleria gruberi* strain Ng 1518, and *Acanthamoeba culbertsoni* strain A-1 were supplied by the Culture Centre for Algae and Protozoa, Cambridge, UK. *N. fowleri* strains NTH, PA14, NHI, and MsT and *N. gruberi* strains Ng-27 and PL200f were obtained from Dr R. A. Robinson of the National Health Institute, Wellington, New Zealand. *N. gruberi* strains BH and BL were obtained from Ms N. Davies, of the Pathology Department of Hamilton Hospital, New Zealand. *Acanthamoeba castellanii* strain 1501, *Acanthamoeba polyphaga* strain P.23, and *Naegleria jadini* strain 0400 were obtained from Dr E. Willaert, Prins Leopold Institute of Tropical Medicine, Belgium. *A. castellanii* strains B and B₁ were isolated from a frozen lake in Oslo, Norway (Brown and Cursons, 1977) and *A. polyphaga* strain NiA from a thermal pool in New Zealand.

All amoebae were grown monoxenically on either amoeba saline agar (Page, 1967) or NM agar

(Fulton, 1970) overlaid with a lawn of *Escherichia coli.*

Strains Ng-27, PL200f, 0400, HB-1, NTH, PA14, NHI, and MsT were grown axenically in CYM medium and strains Ng 1518, BH, and BL were grown axenically in TYG medium (O'Dell and Sevens, 1973). All *Acanthamoeba* spp. were cultured in 4·0% Neff medium (Stevens and O'Dell, 1973).

All strains were subcloned by the plaque method (Fulton, 1970). Cell numbers were estimated to give a total count using a modified bright-line Fuchs Rosenthal haemocytometer.

CELL CULTURE

All cell cultures were maintained in 250-ml flat-bottomed flasks, 10-ml Kimax tubes, or Falcon cell culture petri dishes. The growth medium (GM) consisted of Wellcome Eagles BHK supplemented with 10% tryptose phosphate broth and 10% bovine serum. The monolayer was washed with Eagles BHK and maintained with GM + 2% fetal calf serum. All titrations and plaquing in cell culture were carried out by standard virological techniques.

MOUSE PATHOGENICITY TESTS

Young Swiss-White mice (10–16 g) were anaesthetised by the intraperitoneal injection of 0·15 ml of a 1% v/v nembutal solution. All mice were then inoculated intranasally with 0·1 ml of a 10^6 cells/ml axenic suspension.

SCANNING ELECTRON MICROSCOPY (SEM)

VERO cells were cultured on glass coverslips (diameter 18 mm) placed in plastic petri dishes. After the desired incubation period the cells were fixed in 6% glutaraldehyde in 0·1 M Na-cacodylate-HCl buffer with 0·1 M sucrose pH 7·2 (previously heated to 37°C) for three hours at 4°C. The cells were then washed twice with cold 0·15 M Na-cacodylate-HCl buffer (pH 7·2) and postfixed in 2% OsO_4 in S-collidine buffer (pH 7·4) for 60 minutes. They were then thoroughly washed with distilled water, plunged into liquid air, and freeze-dried in an Edwards Freeze Drier EFO3 (using liquid air as the desiccant) at 10^{-2} torr for four hours. The cells were then fixed onto the stub with silver conductive paint, vacuum coated with gold in a Film Vac. Inc. Mini-Coater, and viewed using a CV/IKSCAN 100 SEM operated at 16 kV.

Results

Table 1 shows that all mouse-pathogenic strains form cytopathic effects (CPE) in VERO cell culture

whereas they are not formed by the non-pathogenic strains. Tables 2 and 3 show the effect of inoculum concentration and the difference in virulence between amoebae passaged in cell culture and those grown in axenic culture.

Typical CPE, such as rounding up of cells (Fig. 3), will develop as early as 8–12 hours, depending on the concentration of the inoculum and the age of the culture. If a lower inoculum concentration and/or older culture is used then the CPE are also slower to appear. This is followed by a steady degeneration of the monolayer with refractility due to granulation and vacuolation of the VERO cells leading to eventual total disruption of the monolayer within 24–48 hours (Figs. 4 and 5).

Animal pathogenicity studies, on the other hand, have been rather less consistent. Table 1 shows that when using either pathogenic Naegleria or Acanthamoeba strains there is a considerable variation in the number of animals that succumb, the incubation period (that is, time between infection and onset of symptoms), and the period of time before death. This variation is due mainly to the individual susceptibility of the animal and the difficulty of being repeatedly precise when using intranasal inoculation.

Discussion

The formation of CPE by PFLA on cell monolayers depends on a number of varying factors such as the virulence of the amoebae, the concentration and age of the inoculum (Stevens and O'Dell, 1974), and the susceptibility of the cell monolayer type. The strains of *N. fowleri* which cause complete disruption of the cell monolayer in two days take two to five times as long to kill mice (Table 1). Similar results are seen with *A. culbertsoni* (Table 1). Using *A. castellanii* and *A. polyphaga*, the time for complete disruption of the cell monolayer is usually one to two days earlier than the time needed to cause the first mouse deaths. The difference in time needed for CPE development is a direct result of the more rapid growth rate of *N. fowleri* in cell culture as compared with *Acanthamoeba* species (Chang, 1971), and of the greater virulence of *N. fowleri* than pathogenic Acanthamoeba (Tables 2 and 3). It is rare for all mice to die after inoculation with *A. castellanii* or *A. polyphaga* (Table 1).

Table 2 shows clearly that for similar sized inocula of *N. fowleri* those amoebae grown in cell culture produce CPE one day earlier than those grown in axenic culture and that for either culture method the reduction of the inoculum concentration by a power of 10 will lengthen the time needed for the development of CPE by one day. This indicates that passag-
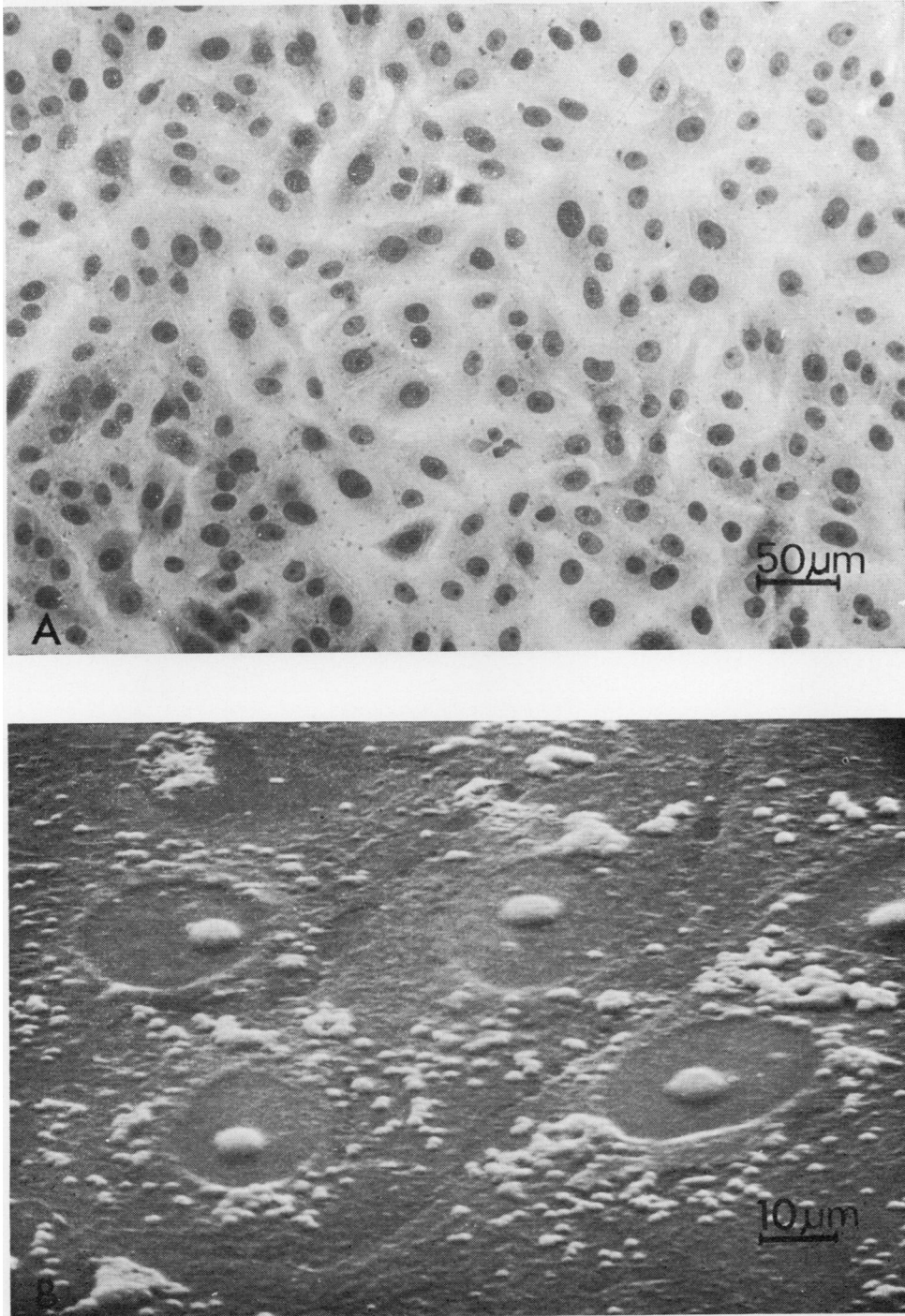
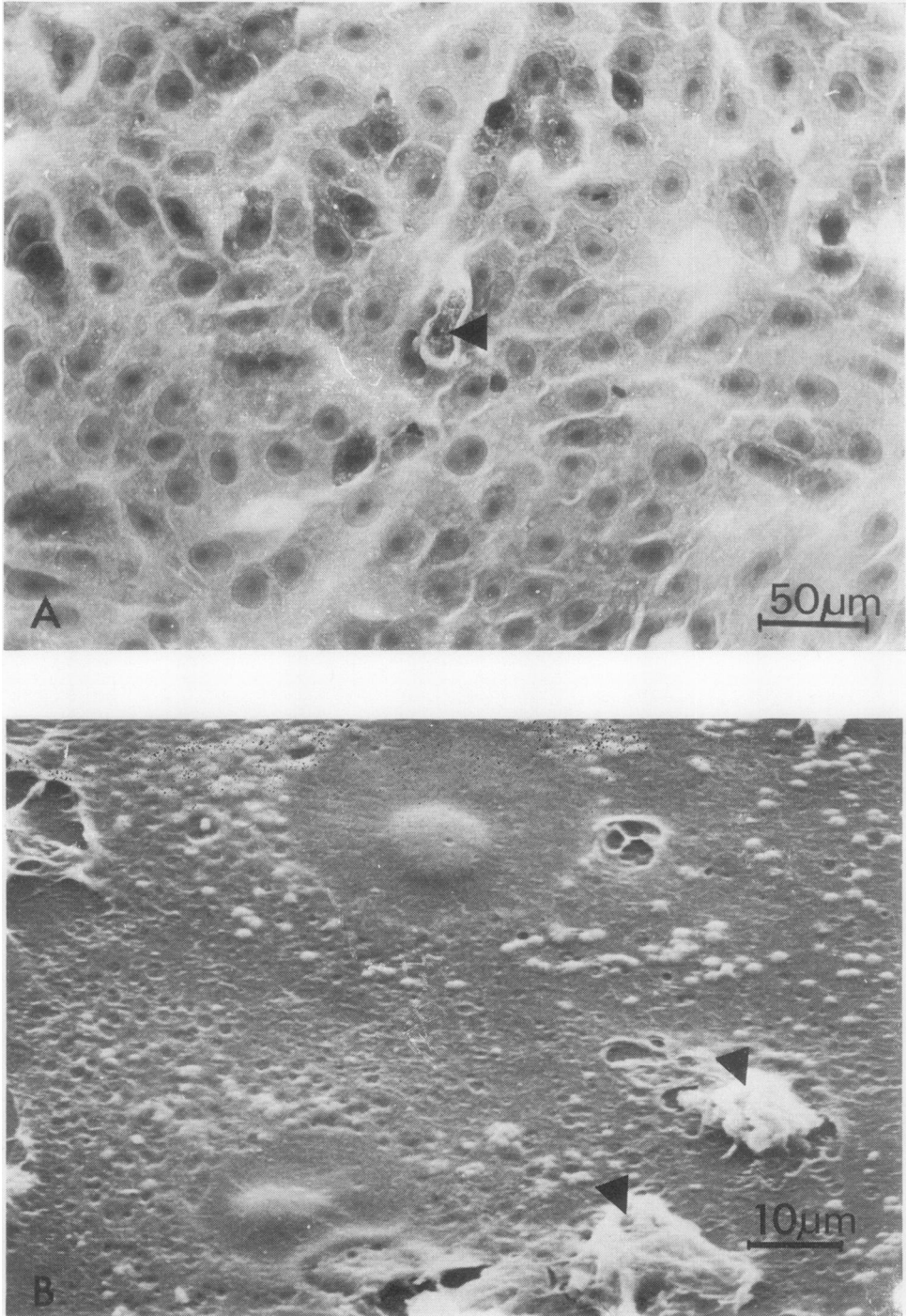Fig. 1 *Control VERO cell monolayer: (A) light micrograph (LM); (B) scanning electron micrograph (SEM).*

Fig. 2   *VERO cell culture showing beginning of cytopathic effects (CPE) (stage I) and amoeba cells*
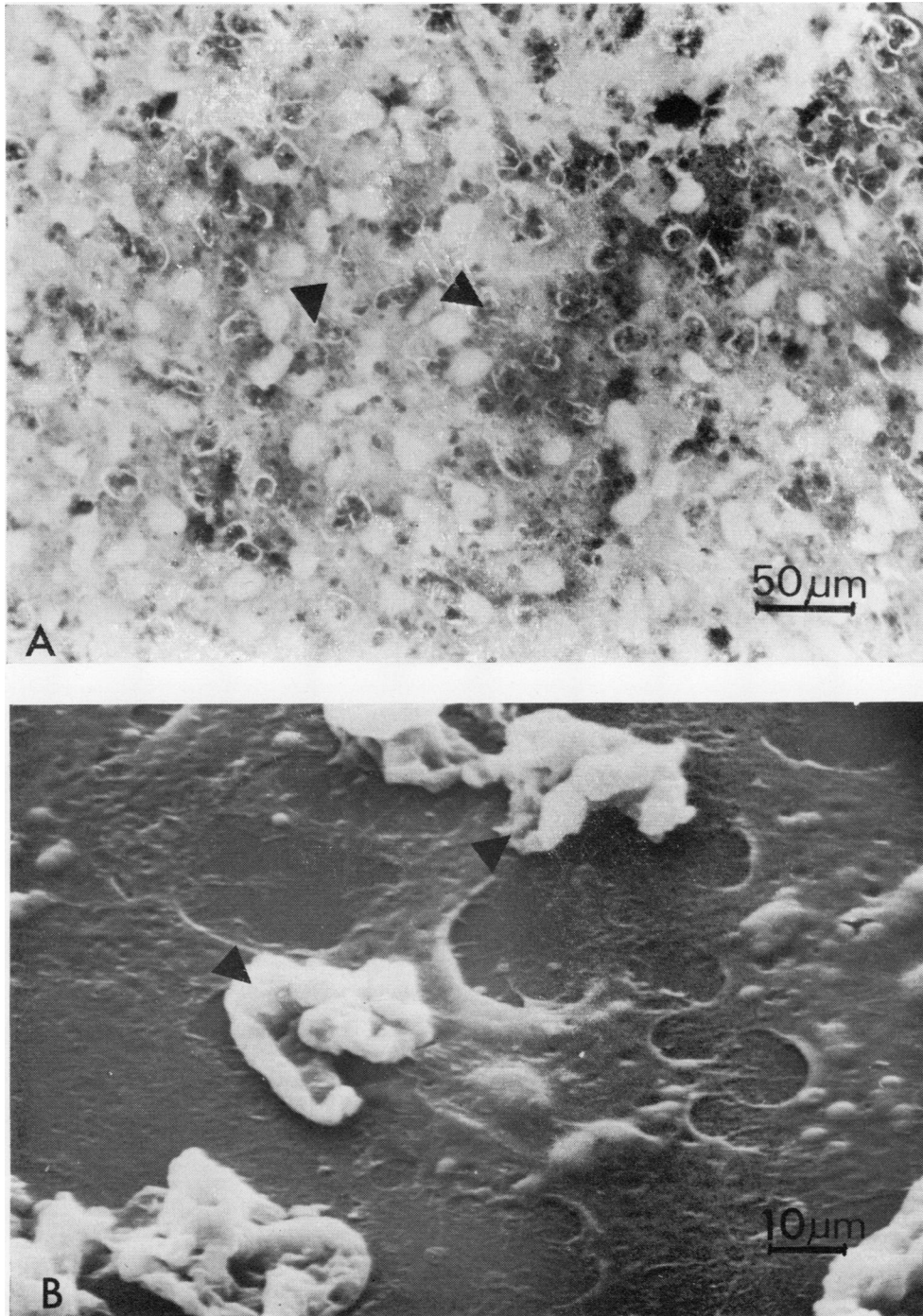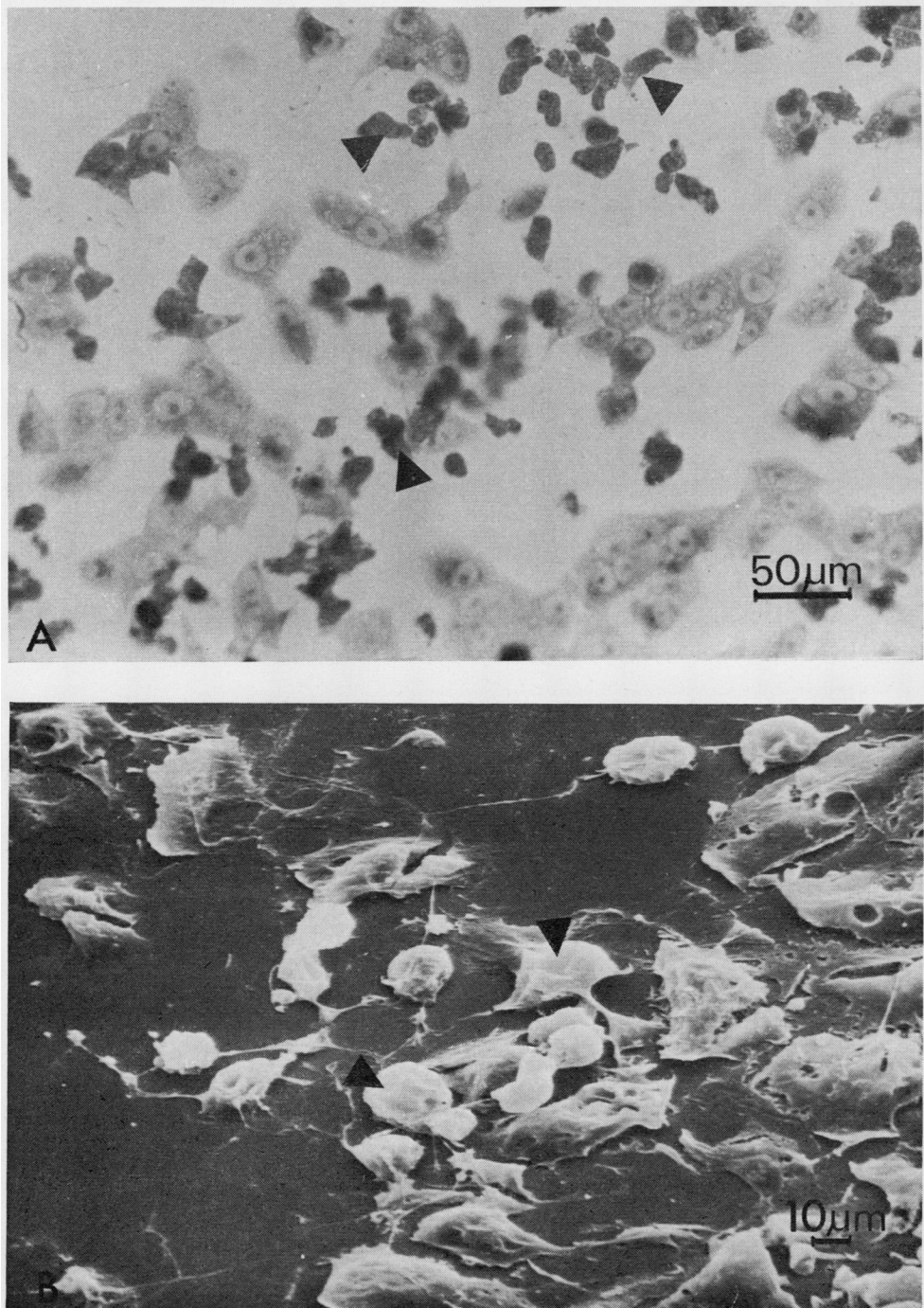(N. fowleri *strain MsT) indicated by arrows; (A) LM; (B) SEM.*

Fig. 3 *VERO cell culture showing pronounced CPE (stage II) with widespread areas of breakdown and numerous amoebae* (N. fowleri *strain MsT*), *some of which are indicated by arrows;* (A) LM; (B) SEM.

Fig. 4   *Pronounced CPE (stage III) showing only isolated islands of VERO cells and many*
amoebae (N. fowleri *strain MsT), some of which are indicated by arrows; (A) LM; (B) SEM.*
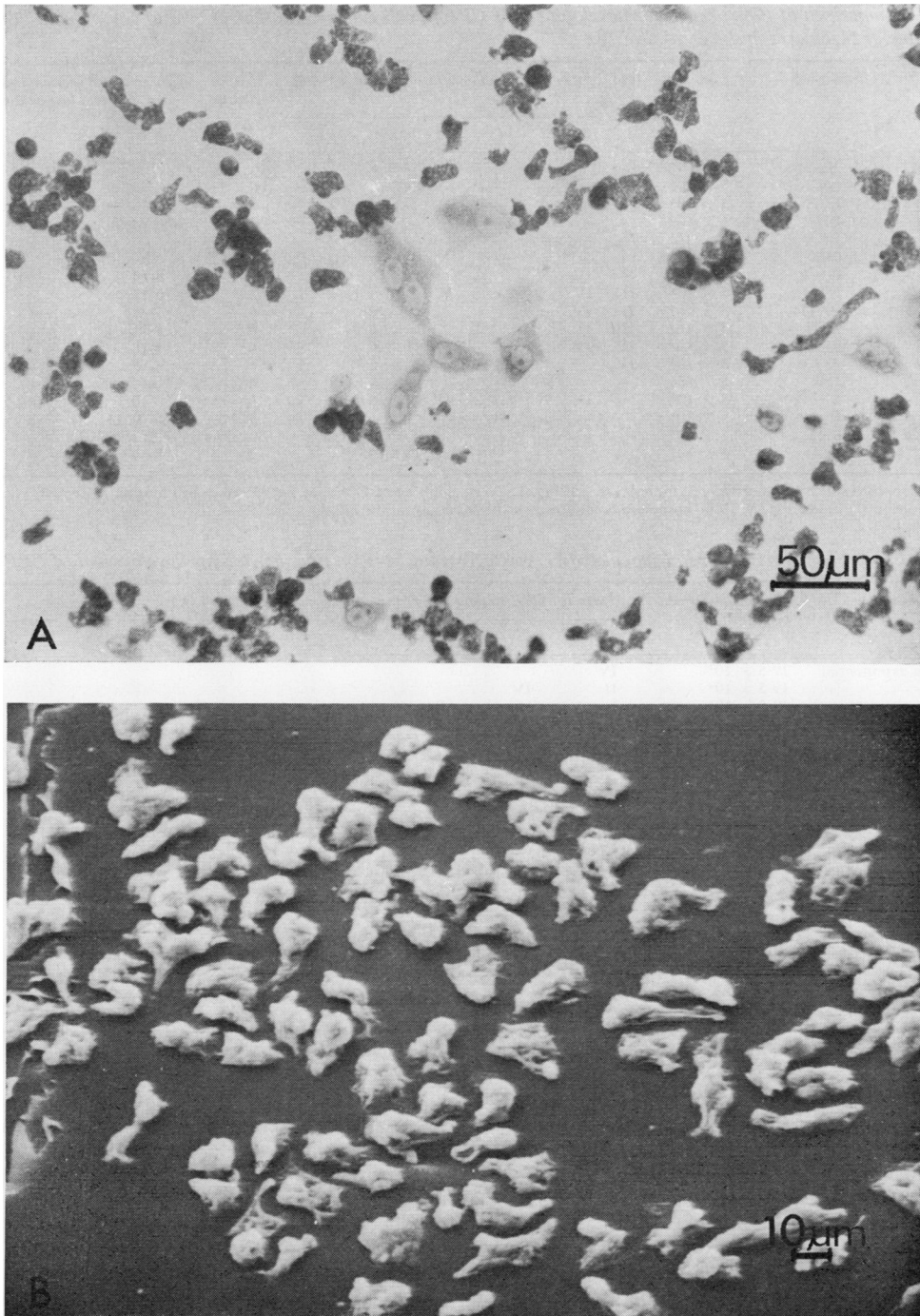
Fig. 5   *Complete breakdown of VERO cell monolayer showing only a few cells: remaining amoebae*
  (N. fowleri *strain MsT*) *dominate the culture* (stage IV); (A) LM; (B) SEM.

Table 1 *Comparison of time needed for development of CPE in cell culture and that needed to cause death in mice using strains of* Naegleria *and* Acanthamoeba

Species	Strain	Conc. of inoculum	No. of days for development of CPE						No. of mice used	No. of deaths	Interval between inoculation and first and last deaths (days)
			1	2	3	4	5	6			
N. gruberi	Ng-27	5 × 10⁴	—	—	—	—	—	—	6	0	
	Ng-1518	5 × 10⁴	—	—	—	—	—	—	6	0	
	PL200f	5 × 10⁴	—	—	—	—	—	—	6	0	
	BH	5 × 10⁴	—	—	—	—	—	—	6	0	Not applicable
	BL	5 × 10⁴	—	—	—	—	—	—	6	0	
N. jadini	0400	5 × 10⁴	—	—	—	—	—	—	6	0	
N. fowleri	HB-1	5 × 10⁴	II	IV					6	5	8-13
	NTH	5 × 10⁴	II	IV					6	2	10-12
	PA14	5 × 10⁴	II	IV					6	6	7- 8
	NHI	5 × 10⁴	III	IV					6	6	6-11
	MsT	5 × 10⁴	III	IV					6	6	5-18
A. culbertsoni	A-1	5 × 10⁴	IV						6	6	4- 5
A. castellanii	1501	5 × 10⁴	—	—	—	—	—	—	6	0	Not applicable
	B	5 × 10⁴	—	I	I	II	IV		6	4	6-10
	B₁	5 × 10⁴	—	—	I	II	IV		6	4	6- 9
A. polyphaga	P.23	5 × 10⁴	—	—	—	—	—	—	6	0	Not applicable
	NiA	5 × 10⁴	—	—	—	I	II	IV	6	2	8-10

(CPE = cytopathic effects; — normal cell monolayer, see Fig. 1; I = beginning of CPE, see Fig. 2; II = pronounced CPE, see Fig. 3; III = very pronounced CPE, see Fig. 4; IV = complete breakdown of the monolayer, see Fig. 5)

Table 2 *Effect of source and size of inocula of* N. fowleri *strain MsT on time needed for development of CPE*

Source of inoculum	Size of inoculum (cells/ml)	Days for CPE to develop							
		1	2	3	4	5	6	7	8
Cell culture exponential 24 h	19·5 × 10⁴	IV							
	19·5 × 10³	II	IV						
	19·5 × 10²	I	II	IV					
	19·5 × 10¹	—	II	III	IV				
	19·5	—	—	—	—	I	III	IV	
Axenic exponential 24 h	18·5 × 10⁴	II	IV						
	18·5 × 10³	—	I	II	IV				
	18·5 × 10²	—	—	I	II	IV			
	18·5 × 10¹	—	—	I	I	II	IV		
	18·5	—	—	—	I	I	III	IV	
Cell culture exponential 24 h	3·4 × 10⁴	IV							
	3·4 × 10³	II	IV						
	3·4 × 10²	I	II	III	IV				
	3·4 × 10¹	—	—	—	II	IV			
	3·4	—	—	—	—	I	II	IV	
Axenic exponential 24 h	3·4 × 10⁴	II	IV						
	3·4 × 10³	—	I	II	IV				
	3·4 × 10²	—	—	—	II	IV			
	3·4 × 10¹	—	—	—	—	I	II	IV	
	3·4	—	—	—	—	—	—	—	—

For explanation of symbols, see Table 1

ing the amoebae through cell culture increases the virulence of the amoebae.

Table 3 shows a similar relationship between inoculum size and source of culture, that is, cell culture or axenic culture, using *A. culbertsoni* although the time pattern of CPE formation is less regular. Using low concentrations of inocula of axenic *A. culbertsoni* there is an apparent loss of virulence even though Acanthamoeba species will grow axenically in Eagles BHK cell culture medium whereas *Naegleria* species will not. This is due to the high salt concentration of the Eagles medium which is amoebicidal to *Naegleria* species. The apparent

loss of virulence of Acanthamoeba when passaged in axenic culture as opposed to mice has been reported by Stevens and O'Dell (1974).

The choice of the cell line does not seem as important as it is in virus infections, amoebae being able to produce CPE in such common cell lines as VERO Hep-2, MDBK, HEL, and HEK. Others have used Pig Kidney, HeLa, PMI, SIRC, and mouse melanoma. Chang (1971), however, states that HeLa cells are unsuitable because both pathogenic and nonpathogenic amoebae produce CPE in this cell line.

The CPE formed by the amoebae on the monolayer appear to be due both to the liberation of a 'cyto-

Table 3 *Effect of source and size of inocula of* A. culbertsoni *strain A-1 on time needed for development of CPE*

History of inoculum	Size of inoculum (cells/ml)	Days for CPE to develop							
		1	2	3	4	5	6	7	8
Cell culture exponential 24 h	30×10^4	IV							
	30×10^3	IV							
	30×10^2	I	III	IV					
	30×10^1	—	I	II	IV				
	30	—	—	—	I	II	III	IV	
Axenic exponential 24 h	30×10^4	IV							
	30×10^3	II	IV						
	30×10^2	—	—	—	II	III	IV		
	30×10^1	—	—	—	—	—	—	—	—
	30	—	—	—	—	—	—	—	—
Cell culture exponential 24 h	3×10^4	II	IV						
	3×10^3	—	—	II	IV				
	3×10^2	—	—	—	—	—	II	III	IV
	3×10^1	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—
Axenic exponential 24 h	3×10^4	—	II	IV					
	3×10^3	—	—	—	I	II	III	IV	
	3×10^2	—	—	—	—	—	—	—	—
	3×10^1	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—

For explanation of symbols, see Table 1

toxic substance' and to actual phagocytosis of the cells by the amoebae, as is seen by using a cell-free filtrate from exponentially growing amoebae passaged in cell culture, and the observation of cell culture debris in the food vacuoles of trophozoites (Chang, 1971, 1974; Visvesvara and Callaway, 1974). In *A. culbertsoni* this 'cytotoxic substance' has been identified as a phospholipase (Visvesvara and Balamuth, 1975), and, more recently, Chang (1976) has also identified a 'phospholipolytic enzyme or factor' in Naegleria. On the other hand, Schuster and Dunnebacke (1974) have described two different intracellular agents in the genus Naegleria, one of which is a virus-like particle (VLP) present only in *N. gruberi* and is not associated with the formation of CPE in cell cultures; the second is an infectious agent present in both *N. gruberi* and *N. fowleri* forming CPE specifically in secondary chick fibroblast cells but not in other cell cultures. Further, this 'Naegleria ameba infectious material' (NAIM) was obtained using a *lysate* from a monoxenic culture as opposed to the cell-free *filtrate* used by us.

The use of cell cultures also avoids the many reported instances of variations in individual susceptibility of laboratory animals (Table 1) (Anderson and Jamieson, 1972; Phillips, 1974; Diffley *et al.*, 1976; Adams *et al.*, 1976). Initially, mice were anaesthetised with ether for the intranasal route but this was changed to nembutal as it was found that residual ether in the nasal cavity lysed amoebae. Although this improved the percentage fatality it is still difficult to quantitate the actual number of trophozoites by this route due to sneezing and swallowing by the mice. Other routes, such as subcutaneous (Culbertson, 1971; Diffley *et al.*, 1976), intravenous, and intraperitoneal (Adams *et al.*, 1976), have also been tried with limited success because of the individual response of the animal.

In our experience, cytopathogenicity of known human and environmental isolates in cell culture has mirrored the pathogenicity in laboratory animals. Chang (1971, 1974), Carter (1972), and Culbertson (1971) have also found this correlation to be experimentally reproducible. Thus cell cultures provide a rapid diagnostic test to indicate the pathogenicity of PFLA and should be used in conjunction with other tests such as the indirect fluorescent antibody test and animal pathogenicity testing. Cell cultures have the advantage over the latter of (*a*) rapidity; (*b*) selecting the more virulent organisms of the population for subsequent use in animal pathogenicity tests; (*c*) providing an axenic source of inoculation, and (*d*) allowing a quantitative estimate to be made of the amoebic population by plaquing in cell culture (Fulton, 1970) (Fig. 6).
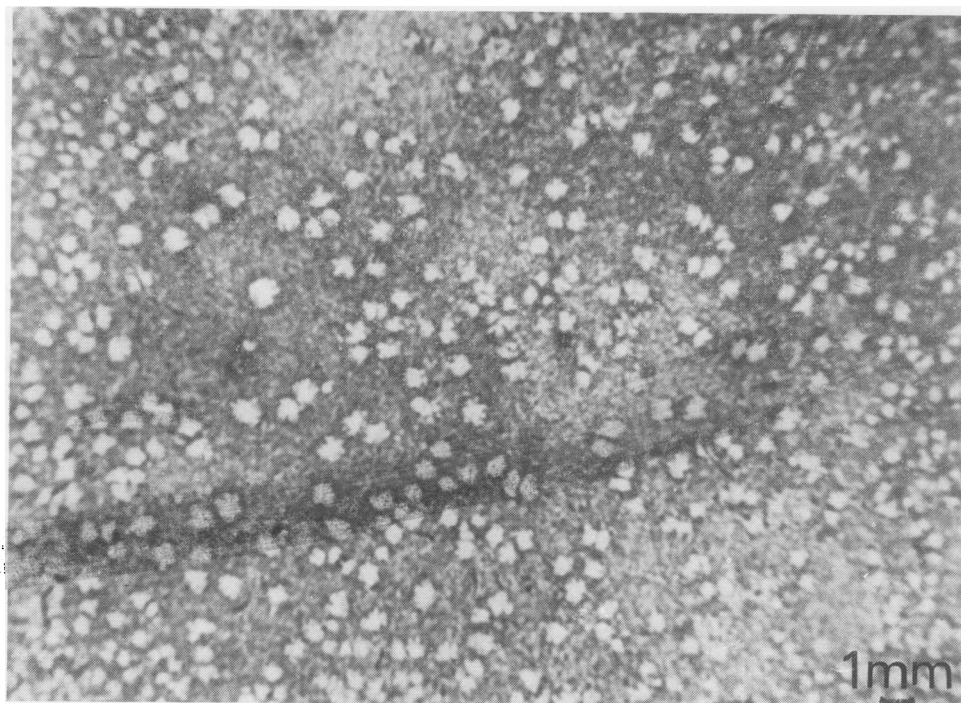
Fig. 6   *Light micrograph of VERO cell monolayer showing plaques cleared by amoebae* (N. fowleri strain MsT). *Each plaque represents one amoeba cell in the inoculum.*

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