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THE IN VITRO AND IN VIVO TESTING OF
CHEMOTHERAPEUTIC AGENTS AGAINST
PATHOGENIC FREE-LIVING AMEBAE

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ABSTRACT

During the last ten years, there has been an increasing awareness of sporadic cases of Primary Amoebic Meningo-encephalitis (PAM) affecting primarily younger age groups and appearing in an acute fulminant form. The earliest positive case (Willaert, 1974) may have been in England in 1909 which shows that the disease has been with us for a long time.

The pathogenic free-living amebae (PFLA), which comprises the genus Naegleria and the genus Acanthamoeba, are the causative organisms of PAM and AM* respectively. PAM is a rapidly fatal disease affecting the central nervous system (CNS), the treatment of which to date has been successful in only a small number of cases, and therefore the continual screening of suitable chemotherapeutic agents against amebae of the Naegleria spp. and Acanthamoeba spp., is of great importance.

AM is also essentially confined to the CNS although it may take the form of chronic granulomata in the liver, spleen, uterus and kidneys (Martinez et al., 1977).

Six chemotherapeutic agents: Amphotericin B, 5-Fluorocytosine, Kanamycin, Oxytetracycline, Tylosine and Levamisole were tested for activity against a non-pathogenic and a pathogenic species of Naegleria and a non-pathogenic and a pathogenic species of Acanthamoeba in axenic culture.

For the Naegleria spp., Amphotericin B and Oxytetracycline were found to be active and the Acanthamoeba spp. were found to be only susceptible to Levamisole.

The synergistic combinations of drugs against the amebae were also investigated in axenic culture. In preliminary trials Kanamycin together with Oxytetracycline showed promise against Naegleria fowleri (MsM) but this was later shown not to be the case. Amphotericin B in combination with 5-Fluorocytosine was also shown not to be synergistic, however Amphotericin B in combination with Oxytetracycline proved to be effective against N. fowleri.

Amphotericin B was combined with 5-Fluorocytosine against A. culbertsoni (A-1) but was not found to be synergistically active.

* Amebic meningitis caused by Acanthamoeba infections.

Levamisole was also tested against N. gruberi (P1200f) and A. castellanii (0.1) at various stages in growth of the amebae (i.e. 24, 48 and 72 hour stock cultures) to determine the effect of using aged amebae. It was found that the age of the stock culture bore no relation to the activity of the drug.

After axenic culture testing, the susceptibility of the pathogenic N. fowleri (MsM) and A. culbertsoni (A-1) to the agents which showed activity, was investigated in a vero cell culture system. For N. fowleri (MsM) the results of axenic culture testing were confirmed, with Amphotericin B and Oxytetracycline protecting the monolayer from the destructive effects of the amebae, both when used singly and at a greater efficiency when added together as a synergistic combination.

Levamisole, although effective to some extent against Acanthamoeba spp. in axenic culture, failed to show any activity against the amebae in vero cell culture testing.

In vivo animal protection studies were then performed using drugs that had been shown either in this or other studies to be effective against either Naegleria or Acanthamoeba spp. Chemotherapeutic agents tested on N. fowleri (MsM) included two imidazoles; Miconazole nitrate and Ketoconazole (previously known as R41,400), as well as Amphotericin B. The synergistic combination of Amphotericin B with either Tetracycline or Oxytetracycline was also investigated.

For A. culbertsoni (A-1), 5-Fluorocytosine, and Polymyxin B were tried both singly and in combination.

These drugs were injected by intraperitoneal (I.P.) and intraventricular (I.vent.) routes. The results were not promising, with none of the drugs offering significant protection even whilst using Amphotericin B which is considered the drug of choice.

The question of adequate drug levels reaching the brain was tested out with two imidazoles, Ketoconazole and Miconazole. Serum samples were assayed against Candida pirapsilosis and C. pseudotropicalis respectively at various time intervals after inoculation with the drug, and a gradual increase and breakdown of the drug in the animal system could then be shown. These results showed that based on in vitro results, the levels of the imidazoles obtained in the serum after the first eight hours after injection, should have been sufficiently high to prevent amebic multiplication.

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CHAPTER ONE: INTRODUCTION

1.1 The History of the Free-living Amebae as Disease Agents

The history of Pathogenic Free-living Amebae (PFLA) of the genera Acanthamoeba and Naegleria has been extensively reviewed elsewhere (Culbertson, 1971; Duma et al., 1971; Chang, 1971, 1974a; Carter, 1972; Cursons, 1974; Cursons and Brown, 1976).

The commonest disease caused by PFLA is Primary Amebic Meningo-encephalitis (PAM) caused by ameba quite different to those traditionally regarded as parasitic in man, and are not ordinarily parasitic in lower animals. They are ubiquitous in the environment and are free-living in water, sewage, soil and other decaying organic matter (Carter, 1972).

Willaert in 1974, tabulated 84 cases from all the continents with the exception of Antarctica. Since then at least 10 additional cases have been reported (Table 1).

Acanthamoeba spp. were the first agents implicated in this disease, but PAM is now known to be caused by a free-living amebae of the genus Naegleria. This was due mainly to the first case prototype of this illness which was described by Fowler and Carter in 1965. In 1968 Carter, Culbertson et al., and Butt et al., showed that the incriminating species of most reported cases belonged to the genus Naegleria, and in 1970 on the basis of morphological, cultural and pathogenicity differences Carter renamed the pathogenic species Naegleria fowleri distinguishing it from the non-pathogenic Naegleria gruberi.

Prior to 1968, all cases of PAM were attributed to the Acanthamoeba spp. which was probably due to the pioneering work of Culbertson et al., (1958, 1959, 1965) who, whilst working on the production of polio vaccine found an ameba which contaminated the cultures of monkey kidney cells. These cultures, when inoculated intracerebrally into mice, produced a necrotic, hemorrhagic meningo-encephalitis that killed mice in 4-7 days. The responsible amebae was identified as an Acanthamoeba and they predicted on the basis of the finding, that this amebae could be capable of producing disease in humans. This ameba was previously considered to be a harmless free-living organism.

The disease caused by PFLA can be divided into two entities (Chang, 1974 (a)):

- a) a swimming-associated acute meningo-encephalitis known as Primary Amebic Meningo-encephalitis (PAM) (Martinez et al., 1977). This is the most important of the two and is caused by Naegleria fowleri (non-pathogen - Naegleria gruberi). Infection is thought to occur in two ways:
 - i) Naegleria contaminated water may be introduced into the upper nasal passages;
 - ii) it may be due to the washing of trophozoites residing in the lower nasal passages of a carrier into the upper nasal area. A pathogenic strain of Naegleria was isolated from a normal healthy carrier (Visvesvara et al., 1974).
- b) a non-swimming-associated chronic meningo-encephalitis (Amebic Meningo-encephalitis (AM) caused by amebae of the Acanthamoeba/Hartmanella group. It is considered that the most probable species is A. rhyodes along with another pathogenic species A. culbertsoni. Amebic Meningo-encephalitis due to the involvement of the Central Nervous System (CNS), appears to be a secondary phenomenon representing metastatic spread from a primary focus in the skin, genitourinary tract or respiratory tract (Martinez et al., 1977). Cutaneous ulceration is a possible point of entry with hematogenous spread to the CNS and lower respiratory tract. Infections in experimental animals have been reported (Martinez et al.,) due to this spread.

Subsequently Acanthamoeba spp. have been indicated in a number of chronic illnesses such as respiratory infections (Martinez et al., 1975), corneal ulceration of the eye leading to blindness (Nadington et al., 1974; Visvesvara et al., 1974) and together with Naegleria spp. in humidifier disease (M.R.C. Symposium, 1977). The reidentification of the etiological agents of the 1968 cases of PAM in New Zealand as N. fowleri species (Cursons and Brown, 1975, 1976) has dismissed the notion of slime moulds in the etiology of PAM (Mandel et al., 1970).

Henceforth, in the text, the nomenclature of Martinez et al., 1977, of PAM for Naegleria infections and AM for Acanthamoeba meningo-encephalitis will be adopted.

1.2 Classification

Study of the basic classification of the small free-living amebae has been stimulated by the discovery of their role in human disease. Carter (1970) accepted the decision of Page to retain the family designation of Vahlkampfiidae (for Naegleria spp.) in preference to the revision by Singh and Das (1970). Recent studies by Fulton (1970) and more recently by Schuster (1975) on the mitosis of Naegleria and related amebae displaying promitosis, have affirmed the lack of validity of details such as "interzonal body" and "polar caps" for use in establishing a higher taxa such as Schizopyrenidae. These points and others are extensively reviewed elsewhere by others (Page, 1974; Chang, 1971; Schuster, 1975; Visvesvara et al., 1975).

In reviewing Acanthamoeba spp. it was clear that they should be moved from the Hartmanellidae and related limax amebae. Page (1967) for one, proposed the replacement of the Acanthamoebae in the Mayorellidae.

In a recent review by Cursons and Brown (1976) the controversy regarding the classification of the PFLA appeared to be settled with the majority preferring Chang's 1971 classification scheme. The identification of isolates involves the exploitation of specific cytological, morphological, physiological, immunological, growth and pathogenicity characteristics in an ordered sequence readily usable by hospital and public health laboratory staff (Cursons and Brown, 1976).

1.3 Occurrence and Distribution

The ability of PFLA to form resistant cysts undoubtedly enables them not only to withstand unfavourable conditions, e.g. the isolation of Acanthamoeba spp. from 2°C (Brown and Cursons, 1977) and from Antarctic soils (Brown et al., pers. comm.), but also to take advantage of the intermittent occurrence of favourable conditions. The PFLA appear to be truly ubiquitous organisms, as isolations have been recorded from a variety of environmental sources such as air (Kingston and Warhurst, 1968), humidifier systems (M.R.C. Symposium, 1977), freshwater, brackish and ocean systems (De Jonckheere et al., 1975; Brown and Cursons, 1977; Stevens et al., 1977a; Wellings et al., 1977), chlorinated swimming and domestic waters (Cerva, 1971a; Anderson and Jamieson, 1977; Cerva and Huldt, 1974), from bottled drinking water (Desmet-Paix, 1974), from a home dialysis unit (Casemore, 1977), from soil (Anderson and Jamieson, 1972; Cursons et al., 1978b), and from sewage (Singh and Das, 1972; Chang, 1974a)

Isolations have also been recorded from cell cultures (Jahnes et al., 1957; Stevens and O'Dell, 1973a; Willaert et al., 1978,) throat and nasal cavities (Elridge and Tobin, 1967; Cerva et al., 1973; Chang et al., 1975), eye infections (Nagington et al., 1974; Visvesvara et al., 1975), gastrointestinal washings (Hoeffler and Rubel, 1974), cold-blooded vertebrates (Frank, 1974), snails (Kingston and Taylor, 1976), and fish (Taylor, 1977).

Temperature and pH are equally tolerated over a wide range with in vitro growth reported up to 45°C (Griffin, 1972) and a pH range of 4.6 to 9.5 (Carter, 1970).

The distribution of the pathogenic species in relation to non-pathogenic ones is still unknown (Cursons, 1978) though in general, non-pathogenic species are more prevalent at ambient temperature in temperate zones. The repeated isolations of PFLA from waters above ambient temperature, i.e. greater or equal to 30°C (De Jonckheere et al., 1975; Stevens et al., 1977a; Wellings et al., 1977; Cursons et al., 1978b), combined with their higher optimum temperature of growth (Griffith, 1972) suggests that the pathogenic amebae are environmentally selected over non-pathogenic amebae in waters above ambient temperature. The source of the pathogenic amebae in these waters is unknown though the fact that Cursons et al. (1978b), and Wellings et al. (1977) have isolated PFLA from the soil, which is the preferred habitat of small free-living amebae (Singh, 1978), makes it possible that the soil acts as a reservoir of pathogens in the same way as it does for *Cryptococcus*, and that contamination occurs via run off after rain (Cursons, 1978).

1.4 Pathogenicity

The invasion of organs and tissues by PFLA is now well documented (Culbertson et al., 1959, 1968, 1972; Carter, 1968, 1970, 1972; Callicot et al., 1968; Chang, 1971, 1974a & b, 1976; Culbertson, 1971; Martinez et al., 1973, 1975, 1977; Visvesvara and Balamuth, 1975; Wong et al., 1975 a & b). The CNS invasion by *Naegleria* spp. occurs primarily via the nasal mucosal epithelium, mainly due to the pathological condition of the cribriform plate and subjacent nasal passages (Culbertson, 1971; Carter, 1972) and this has been verified experimentally by Martinez et al. (1973). Using mice they showed that amebic invasion occurs through the disruption of the olfactory mucosa, penetration into the submucosal plexus, probably by phagocytosis by the amebae of the sustentacular cells of the olfactory

neuroepithelium, and finally through the cribriform plate to the CNS.

In cases of *Acanthamoeba* meningo-encephalitis, the involvement of the CNS appears to be a secondary phenomenon representing metastatic spread from a primary focus in the skin, genitourinary or respiratory tract (Martinez et al., 1975, 1977; Culbertson, 1971). Martinez et al. (1975) reported lower respiratory tract infections in experimental animals.

AM due to *Acanthamoeba* spp., appears to be due to an opportunistic infection of the CNS. AM occurs in patients who are chronically ill, debilitated or in those whose cell mediated immune responses have been impaired as a result of either underlying systemic disease or its treatment by immunosuppressive methods (Kernohan et al., 1960; Jager and Stamm, 1972; Robert and Rorke, 1973; Bhagwandeem et al., 1975).

Acanthamoeba infections of sites with reduced accessibility to the immune system (e.g. the eye) also demonstrates the opportunistic nature of these infections. Isolations of *Acanthamoeba* from the cornea of the eye (Nagington et al., 1974) were shown to be of low virulence and infection only resulted after damage to the cornea (Visvesvara et al., 1975).

Once CNS invasion has occurred, destruction of the surrounding tissue is thought to be brought about by a combination of phagocytosis and pinocytosis of host tissue by *N. fowleri*, and solely by pinocytosis in the case of *A. culbertsoni* (Visvesvara and Callaway, 1974; Maitra et al., 1974, 1976). The extensive reports of the possession of lysosomal and hydrolytic enzymes is reported elsewhere (Bowers and Korn, 1973; Martinez et al., 1975; Chang, 1976; Maitra et al., 1976; Cursons and Brown, 1976), and it is also speculated that the levels of the cytopathic enzymes produced may explain the degrees of virulence among the *Acanthamoeba* and the *N. fowleri* isolates (Cursons, 1978; Culbertson, 1971; De Jonckheere and van de Voorde, 1977b).

1.5 Immunity

Many authors have pondered over the low incidence of PAM and AM cases with regards the ease and frequency of isolation of pathogenic PFLA from the environment (Anderson and Jamieson, 1971; Cursons et al., 1977b, 1977; John et al., 1977; Wellings et al., 1977; Haggerty and John, 1978). This has led many to speculate on the existence of probable host related susceptibility factors and the demonstration of specific

antibodies to free-living amebae in human sera has been reported (Chang and Owens, 1964; Edwards et al., 1976; Cursons et al., 1977; MRC Symposium, 1977).

Adams et al., (1976) reported that mice surviving a primary intravenous injection of N. fowleri were subsequently resistant to further challenge by the same route with a dose of amebae that produced a uniformly fatal disease in untreated control mice. It was further demonstrated by this group that mice immunized with live or formalized N. fowleri or live N. gruberi either subcutaneously, intraperitoneally, intravenously or intramuscularly were significantly protected against a subsequent challenge with N. fowleri (John et al., 1977). The role of cell mediated immunity (CMI) in resistance to infection by N. fowleri was reported by Diffley et al. (1976), who demonstrated that guinea-pigs surviving a normally fatal challenge with N. fowleri, exhibited a delayed hypersensitivity when tested intradermally with a soluble fraction derived from N. fowleri (Cursons et al., 1977). Thong (1978) stated that protective immunity to PAM could be transferred to syngeneic mice by immune sera but not by immune spleen cells. The immunity may be related to agglutinating antibodies demonstrated in immune sera or antitoxic antibodies in immune sera may be the active principle. These all tend to support the hypothesis that unwitting exposure to the more ubiquitous non-pathogenic N. gruberi may immunize against N. fowleri and the same may also occur with Acanthamoeba spp. The fact that some underlying immunity exists was demonstrated by Wong et al. (1975 a & b) who demonstrated that primates were apparently immune to intranasal or intravenous inoculations of N. fowleri or A. culbertsoni unless on immunosuppressive drugs. However intrathecal inoculations were shown to cause amebic meningo-encephalitis. Culbertson has shown that mice immunized with Acanthamoeba spp. are resistant to intranasal challenge with A. culbertsoni but was unable to show the same with Naegleria spp.

1.6 Control Measures

Free-living amebae are widely dispersed in the environment and the fact that they can be isolated from chlorinated domestic and swimming waters (Cerva, 1971a; Anderson and Jamieson, 1972; Cerva and Huldt, 1974; De Jonckheere and van de Voorde, 1976), as well as untreated recreational waters has led to an expression of concern by public health authorities over the possible contraction of PAM or AM via these sources.

Cerva (1971a), after reviewing 16 fatal cases of PAM from an indoor chlorinated swimming pool stated that, there will always be the constant presence of limax amebae even under the strict observations of

all routine safety measures applied to swimming pools and water systems. This was supported by a reported case of PAM in South Australia by Anderson and Jamieson (1972), in which the victim contracted the disease from domestic bath water, and that super chlorination to 10mg.l⁻¹ failed to eradicate Naegleria from the contaminated pool. However, Lyons and Kapur (1977) in a survey of 30 halogenated public swimming pools concluded that the low amebic densities (less than one per litre), in the majority of pools illustrated that these amebae could be adequately controlled by proper pool maintenance. The possession of resistant cysts however complicates the disinfection process.

Derreumaux et al. (1974) demonstrated that 0.5mg.l⁻¹ of HOCl, the active disinfecting component of chlorine disinfection was able to eradicate both Naegleria and Acanthamoeba spp. De Jonckheere and van de Voorde (1976), showed that an initial concentration of chlorine between 0.5 - 1.0mg.l⁻¹ was cysticidal for Naegleria spp. but that Acanthamoeba culbertsoni cysts were not inactivated by levels up to 40mg.l⁻¹.

In a study of alternative disinfectants by Cursons et al., (1978b) it was shown that deciquam 222, chlorine, chlorine dioxide and ozone all possessed potential disinfecting properties for PFLA, but at higher levels than those for disinfecting bacteria. Deciquam 222 was found to be the most effective followed by chlorine, chlorine dioxide and ozone, but the final choice of disinfectant must depend on the physical and chemical properties of the water to be treated.

1.7 Diagnosis

Early diagnosis and treatment along with careful intensive care treatment therapy is extremely important in the treatment of infections due to PFLA; more in those caused by Naegleria spp. The survival of a nine year old female in Torrance, California (Siedel et al., pers. comm. 1978) and that of a fourteen year old male in Australia (Anderson and Jamieson, 1972) could be attributed to this. Fluid restriction, management of cerebral edema and other complications of amebic meningo-encephalitis are all important in the care of these patients (Siedel et al., pers. comm. 1978).

Infections due to Naegleria spp. are usually characterized by a previous history of swimming in freshwater some 7-14 days before expressing typical meningitis symptoms (Cursons et al., 1977; Carter, 1972; Chang, 1974a). The symptoms include severe headache (usually frontal), sore throat, nausea, vomiting, fever (39-41°C) accompanied

by a stiff neck. Clinical isolation of amebae can be routinely done by cultivation of Cerebral Spinal Fluid (CSF), brain tissue or nasal discharge on Page's Ameba Saline Agar spread with live E. coli or E. cloacae; by axenic CYM culture; or by passaging of suspected material through cell culture, at 37-45°C (Cursons et al., 1978). The examination of CSF is still probably the most routine method of diagnosing general meningitis. The differences between amebic and bacterial meningitis are slight and although in positive amebic cases there tend to be a predominance of neutrophils in the CSF, a high protein concentration and low sugar levels, complete diagnosis relies on finding amebae in the fluid and the further cultivation of these for complete diagnosis. Species identification can then be achieved by a method outlined by Cursons and Brown (1976).

In post-mortem diagnosis, a degree of encephalitis is invariably present. Severe brain swelling and redness, combined with purulent and haemorrhagic exudate containing numerous amebae is more extensive on the ventral surface of the cerebrum or cerebellum and over the brain stem. Amebae are also numerous in the olfactory nerve bundles which are virtually destroyed by purulent inflammation (Carter, 1969, 1972). The grey matter of the cerebral hemispheres and cerebellum shows variable sized lesions which tend to be haemorrhagic and quite soft when they are large (Culbertson, 1971). Purulent meningitis is usually inconspicuous and confined to the antero-basal aspects of the brain, and it is only rarely that one can find inflammation or amebic invasion in the posterior cerebral hemispheres, brain stem or cerebellum, and never in the spinal cord (Carter, 1969, 1972).

The Indirect Immuno Fluorescent Antibody (IFAB) technique applied to hydrosoluble protein extracts of either Naegleria or Acanthamoeba spp. is a valuable tool in the identification of species. It can also be applied to identify amebae in brain sections of suspected or proven patients, though is a time consuming process and is not recommended for routine laboratory practice. Antisera can be produced in rabbits and can be made species specific by suitable absorption methods. IFAB methods can also be used to provide rapid screening methods for detection of PFLA in swimming pools, tap and other domestic and recreational water supplies.

Immunoperoxidase methods have been used to demonstrate both Naegleria and Acanthamoeba spp. in brain sections of patients who have died from PAM and AM respectively by Culbertson (1975) and Cursons et al., (1976). This is a method that may be shown to be more valuable

in the future than immunofluorescence techniques. It has certain advantages over IFAB in that permanent preparations can be made, no specialized equipment is necessary and clear definitive staining of tissue elements results (Culbertson, 1975).

Acanthamoeba meningitis infections are difficult to diagnose even in advanced cases due to the lack of specific symptoms and the apparent lack of amebae in the CSF (Chang, 1974a). There is usually a history of poor health and immunological incompetence with few patients giving a past history of swimming. The onset is slow (>10 days) and insidious, with the lung, brain and kidneys being infected (Martinez et al., 1976). Acanthamoeba infections may initially produce a severe bronchopneumonia, the organisms then disseminating and reaching the CNS via the bloodstream (Marino, 1975).

Post-mortem diagnosis relies on the presence of superficial lesions in the grey matter with granulomatous inflammation, and the presence of trophozoites and double walled wrinkled cysts in apparently normal tissue bordering the lesion (Chang, 1974a; Carter, 1972; Culbertson, 1971; Hoffmann et al., 1978). Many authors regard this as diagnostic of Acanthamoeba infections.

In the case of eye infections reported by Nagington et al. (1974) and Jones et al. (1975), positive diagnosis was possible by taking corneal scrapings, with subsequent isolation and identification of Acanthamoeba spp.

1.8 PAM Cases and Their Treatment

Since Willaert published the extensive review of world-wide cases due to PAM in 1974, there have been at least ten additional cases reported (Table I). Symmers (1969) reports a possible earliest case dating back to 1909. A later case reported by Derrick et al. (1948) was originally thought to be due to Iodamoeba butschlii, but was later proven by fluorescent antibody staining to have been caused by N. fowleri (McMillan, 1977). The confusion in this case arose through the patient having widespread alimentary and systemic invasion as well as the typical pattern of cerebral invasion by morphologically identical amebae, thought to be caused by starvation of the patient, perhaps by reducing his gastric activity, bile secretion and amebicidal serum factor (Carter, 1970). The reidentification of the etiological agents of the 1968 cases of PAM in New Zealand as N. fowleri (Cursons and Brown, 1975; Cursons et al., 1967a) has dismissed the notion of slime moulds being involved in

COUNTRY	YEAR	NUMBER OF CASES	CAUSATIVE ORGANISM	DIAGNOSIS	TREATMENT	OUTCOME	REFERENCE
NEW ZEALAND	1974	1	<u>N. fowleri</u> (MsT)	isolation from CSF	Penicillin Ampicillin Amphotericin B	died	Cursons <u>et al.</u> , 1976b
	1978	1	<u>N. fowleri</u> (MsM)	isolation from CSF	Amphotericin B	died	Cursons <u>et al.</u> , pers. comm., 1978
U.S.A.	1974	1	<u>N. fowleri</u> (Lovell)	isolation from CSF	Unknown	died	De Jonckheere, 1977
	1974	1	<u>Acanthamoeba</u> sp.	IFAB	Steroids Penicillin	died	Martinez <u>et al.</u> , 1977
	1975	1	<u>Acanthamoeba</u> sp.	IFAB post-mortem	Unknown	died	Hoffman <u>et al.</u> , 1978
	1978	1	<u>Naegleria</u> sp.	isolation from CSF	Amphotericin B Miconazole Rifampin	survived	Seidel <u>et al.</u> , pers. comm., 1978
VENEZUELA		1	<u>A. culbertsoni</u>	IFAB	Steroids	died	Martinez <u>et al.</u> , 1977
PERU		1	<u>A. castellanii</u>	IFAB	Steroids Antibiotics	died	Martinez <u>et al.</u> , 1977
ZAMBIA	1972	1	<u>Acanthamoeba</u> sp.	post-mortem	Antibiotics Amphotericin B	died	Bhagwandeem <u>et al.</u> , 1975
KOREA	1958	1	<u>Acanthamoeba</u> sp.	post-mortem	Penicillin Streptomycin Chloramphenicol	died	Ringsted <u>et al.</u> , 1975

Table I: Cases of Primary Amebic Meningo-encephalitis Reported After 1974 (modified from Cursons, 1978)

COUNTRY	YEAR	NUMBER OF CASES	CAUSATIVE ORGANISM	TREATMENT	REFERENCE
UGANDA	1968	1	<u>Naegleria</u>	Metronidazole - Emetine - Penicillin Chloroquine.	Grundy & Blowers, 1970
U.S.A.	1967	1	<u>A. astronyxis</u>	Ampicillin Penicillin - G	Callicott <u>et al.</u> , 1968
	1978	1	<u>Naegleria</u>	Amphotericin B Miconazole Rifampin	Seidel <u>et al.</u> , pers. comm. 1978
INDIA	1970	2	<u>Naegleria</u>	Streptomycin Isonicotinhydrosine Sulphadexanathosone Amphotericin B	Pan & Ghosh, 1971
	1973	3	<u>N. fowleri</u>	Unknown	S.R. Das, pers. comm. to Willaert(1974)
ENGLAND	1969	2	<u>Naegleria</u>	Antibiotics Sulphadiazine Amphotericin B	Apley <u>et al.</u> , 1970
AUSTRALIA	1971	1	<u>N. fowleri</u>	Amphotericin B Sulphadiazine	Anderson & Jamieson, 1972

Table II: Probable and Definite Survivors of Primary Amebic Meningo-encephalitis

the etiology of PAM (Mandel et al., 1970).

The drug treatment of PAM has been very discouraging with Willaert's summary (1974) providing information of only ten possible survivors of the disease. The Californian case of Seidel et al. (1978) brings the world total to eleven cases (Table II). In the earlier cases, where the amebic nature of the disease had not been suspected, treatment consisted only of antibacterial agents such as sulpha-drugs, penicillin, streptomycin, tetracyclines and chloramphenicol (Fowler and Carter, 1965; Butt et al., 1968; Cerva and Novak, 1968; Dos Santos, 1970; Van den Driessche et al., 1973). However, even in later cases where the antiprotozoal drugs emetine, chloroquine and metronidazole were often used, the course of the disease was not affected in the slightest (Carter, 1968, 1970, 1972; Duma et al., 1971) except in the unproven case of Grundy and Blowers (1970) in which survival was attributed to chloroquine. Naegleria were supposedly isolated from the CSF but failed to survive for any length of time in culture and subsequently were not positively identified. The patient also presented atypical symptoms and treatment consisted of metronidazole, emetine, penicillin sulphane and chloroquine.

The in vitro activity of antibacterial agents against pathogenic Naegleria has been extensively reviewed by many (Carter, 1969; Mandel et al., 1970; Prasad, 1972; Thong et al., 1977; Lee et al., 1979, Donald et al., 1979). Of the antiprotozoal drugs, emetine hydrochloride was shown to be effective in vitro against N. fowleri (Carter, 1969; Prasad, 1972; Das, 1975) although it does not protect animals from the disease (Culbertson et al., 1968), probably due to its inability to pass the blood-brain barrier (Parmer and Cottrill, 1949). Chloroquine and metronidazole have also been shown to be ineffective both in in vitro and in vivo studies (Carter, 1969; Mandel et al., 1970; Duma et al., 1971).

Amphotericin B was the only drug to appear promising in the early 70's, and as can be seen in Table II, it was used in the treatment of all survivors except the unproven case of Grundy and Blowers (1976) and Callicott et al., (1968). Amphotericin B is an antifungal polyene antibiotic and in vitro tests have shown it to be very effective against Naegleria spp. (Carter, 1969; Mandel et al., 1970; Duma et al., 1971; Schuster and Rechthand, 1975; Visvesvara and Balamuth, 1975; Duma and Finley, 1976; De Jonckheere and van de Voorde, 1977; Donald et al., 1979) and to show in vivo promise (Culbertson et al., 1968; Carter, 1969; Das 1971; Thong, 1978, 1979).

Carter (1969) suggested that amphotericin B be tried in the treatment of PAM by simultaneous intravenous and intraventricular administration. The doses recommended were: 0.25mg.kg^{-1} IV and 1.0mg into the cerebral ventricles (I.vent.) in the first 24 hours which were as high as he dared propose due to the highly toxic nature of the drug. Carter (1972) also suggested using sulphadiazine as well as amphotericin B initially, in case the amebae should prove to be Acanthamoeba. These amebae have shown to be resistant to both these drugs in vitro (Casemore, 1970; Chang, 1971; Visvesvara and Balamuth, 1975; Duma and Finley, 1976; Nagington and Richards, 1976; Donald et al., 1979), but there is evidence to show that they are affected by sulphadiazine in vivo (Culbertson et al., 1965). Subsequently the treatment was tried on two patients in the U.S.A. (Duma et al., 1971) who were in the early stages of the disease and should have responded. The first patient (patient 3, Duma et al., 1971) was given 1.5mg of amphotericin B through a ventricular tap which was repeated 16 hours later. 10mg amphotericin B was also administered I.vent. together with 10mg dexamethasone. The patient was also given 400mg metronidazole (orally); 200mg chloroquine base and 4mg dexamethazone intramuscularly (IM) every 6 hours. However, 72 hours after admission he became shocklike, respiration ceased and he died. The second patient (patient 4, Duma et al., 1971) received similar treatment though he died 66 hours after admission.

Carter (1972) reported similar findings to Duma et al (1971) in two patients that had been treated in the same way (seventh and ninth patients Table III, Carter, 1972). There was also the added difficulty in getting the drug into the swollen brains by the intraventricular method.

Apley et al (1970) described three cases of PAM in Britain, two of which were diagnosed presumptively because of association with the fatal proven case. They had the same early symptoms, however, neither actually developed the convincing signs of meningitis. N. gruberi was cultured from the CSF of the child who died and from only one of the others. Amphotericin B was administered to the fatal case after finding amebae in the CSF. It was given IV in one daily dose of 0.25mg.kg^{-1} given over three to four hours. This was increased to 1mg.kg^{-1} over one week, but the patient died on the sixteenth day after admission. It is interesting to note that on the seventh day after admission amebae were seen in the CSF though many appeared to be dead. By the eleventh day after admission, the CSF contained no amebae. It is also interesting that the drug was given by the intravenous route only, and yet produced

high levels in the CSF, apparently destroying most of the amebae in the CNS. The patient's survival had been notably prolonged and Carter (1972) postulated that maybe IV treatment on its own, but at a higher dosage rate, may be successful in further cases. This was in fact proved in a later case (Anderson and Jamieson, 1972).

The second British case, a brother of case one, was admitted to hospital two days after case one, complaining of a headache and sore throat with neck pains. In view of patient one, amphotericin B and sulphadiazine treatment was begun although the CSF was clear. By day seven he was symptom clear though on day eight they returned and although the CSF was clear, some amebae were cultured which appeared to be similar to those from case one. Amphotericin B was given, $0.25\text{mg}\cdot\text{kg}^{-1}$ IV over four hours increasing to $0.75\text{mg}\cdot\text{kg}^{-1}$ after four days for a total of 10 days after which the CSF was clear and no amebae were cultured. He was discharged symptom free.

The third case was admitted to hospital six days after case one. He complained of sore throat, headache, vomiting and abdominal pain although CSF appeared normal. He was given amphotericin B and sulphadiazine though signs of drug toxicity were noted after four days and the treatment was stopped. It was on day eight that the growth of amebae from case two was reported and although the patient was well, amphotericin B treatment was recommended at $0.25\text{mg}\cdot\text{kg}^{-1}$. He was discharged after fourteen days, symptom free with no amebae having been isolated at any time (Apley *et al.*, 1970). "Case three must be considered to be only doubtfully infected with Amebae" (Apley *et al.*, 1970). Griffin (1976) has disputed the diagnosis of *Naegleria meningoencephalitis* in cases one and two and believes that *Acanthamoeba* were in fact the ameba involved, and that sulphadiazine was responsible for the treatment of case two and the prolonged survival in case one. He also considers that the level of sulphadiazine in the CSF prevented the growth of amebae in culture.

Pan and Ghosh (1971) reported the survival of two children (aged 6 months and 3 years) with CNS infections of slow onset (3-5 months). CSF samples showed "motile amebae with thin pseudopods" and, although no strains were isolated, treatment was with amphotericin B, sulphadiazine and intrathecal steroids. These two cases are considered inconclusive in the nature of the etiological agent involved and the effective agent in their treatment (Donald, 1979).

The first successful treatment of N. fowleri PAM was that reported by Anderson and Jamieson (1972). A fourteen year old boy from Queensland was in the fourth day of illness and comatose by the time treatment was begun. N. fowleri was cultured from the CSF, in which they could be plainly seen. Amphotericin B was given at a dose of 1mg.kg^{-1} per day IV, as well as penicillin, -ampicillin and sulphadiazine which he had been having for three days previously. He was afebrile and talking rationally within two days. After five days the CSF white cell count had dropped but amebae were still seen, therefore amphotericin B was given intrathecally (IT) and later I.vent. in small doses (0.1mg on alternate days). The fluid gradually cleared and he was discharged from hospital without any neurological defects.

The second successful treatment of a N. fowleri PAM case is that of a nine year old female from Torrance, California who showed typical symptoms of meningo-encephalitis three days before admission to hospital (Seidel et al., pers. comm.). Routine CSF cell count procedures revealed organisms with ameboid movements and the following medications were given: amphotericin B - 1.5mg IT and 1mg.kg^{-1} IV; sulphadiazine 50mg.kg^{-1} IV; chloramphenicol 25mg.kg^{-1} IV and penicillin 3.4×10^5 units IV. The patient was then transferred to Harbor General Hospital where she was in a coma on admission but responsive to pain and tactile stimulation. The following treatment was administered:

- (i) Amphotericin B was given IV at a dose of $1.5\text{mg.kg}^{-1}.\text{day}^{-1}$ given in two doses daily for three days after which it was decreased to $1\text{mg.kg}^{-1}.\text{day}^{-1}$ given in a single daily dose for six days.
- (ii) Amphotericin B was also given IT at $1.5\text{mg}.\text{day}^{-1}$ for two days after which it was decreased to 1.0mg every other day for eight days. This was administered through a lumbar intrathecal catheter.
- (iii) Miconazole was given IV at a dose of $350\text{mg.m}^{-2}.\text{day}^{-1}$ given thrice daily for nine days.
- (iv) Miconazole IT at $10\text{mg}.\text{day}^{-1}$ for two days then 10mg every other day for eight days.
- (v) Rifampin was given orally at a dose of $10\text{mg.kg}^{-1}.\text{day}^{-1}$ thrice daily for nine days.

Sulphadiazine (IV - $4\text{gms}.\text{day}^{-1}$) was continued for three days until studies confirmed the diagnosis of Naegleria meningo-encephalitis. Penicillin and chloramphenicol were continued for three days until CSF

cultures were shown to be negative for bacteria. Dexamethasone and diphenylhydantoin were given for increased intracranial pressure and seizure activity respectively. The patient stabilized clinically over the first forty eight hours. Gradually over the next month of hospitalization her mental status improved and no significant neurological deficits were noted at discharge (Seidel et al., -pers. comm).

In other reported cases of PAM where there was proof of N. fowleri infection, and where amphotericin B was given as a treatment, the course of the disease was often too advanced to see any effect (Van Den Driessche et al., 1973; Donald, 1979).

Amebic meningitis due to Acanthamoeba are a lot less common than those of Naegleria probably due to the need for some predisposing factor (Martinez et al., 1977; Kernohan et al., 1960; Jager and Stamm, 1972; Bhagwandeem et al., 1975). Callicott et al. (1968) reported a survival due to A. astronyxis which was isolated from the CSF although the authors were unable to provide evidence as to whether the disease was in fact due to Acanthamoeba.

Several cases of Acanthamoeba infection have been reported though only after post-mortem examination where the brain sections were stained by indirect immunofluorescent antibody techniques (Ringsted et al., 1976; Martinez et al., 1977; Hoffmann et al., 1978; Willaert, 1978).

A possible case was reported by Kenney (1971) in a patient hospitalized for acute gastritis of unknown origin. Compliment fixation tests revealed no antibodies to Entamoeba histolytica though did reveal some to A. culbertsoni which rose over the next two months. Clinical examination failed to reveal any symptoms of cerebral involvement and the patient refused a spinal tap. The patient was put onto antiamebic treatment consisting of dehydro-emetine and chloroquine (IM). Compliment fixation tests two months later showed that the serum titre had decreased.

The only human Acanthamoeba infections positively diagnosed during life were those in the eye. Nagington et al. (1974) repeatedly isolated Acanthamoeba from two English patients with corneal ulcers. Warhurst and Thomas (1975) identified the amebae as A. castellanii and A. polyphaga. In one case, chloramphenicol, idoxuridine, 3-fluor-thymidine, methicillin, gentamicin and later sulphadiazine were tried without any effect. After six months, because of corneal ulceration, pain and loss of vision, a corneal graft was performed which was rejected.

The other infection was in a 59 year old farmer with an identical condition which required enucleation of the eye after one year. Treatment was in this case, chloramphenicol, acetylcysteine, 3-fluorothymidine and clotrimazole.

At the same time as the above eye infections, Jones et al. (1975) cultivated A. polyphaga from corneal ulcers of two patients in Houston, Texas. They reported suppression of the amebae with paromomycin. It seems that these infections may not be so rare, and in cases of chronic corneal ulceration, amebic infection should always be considered (Nagington et al., 1974).