Cryptococcal meningitis in Hong Kong

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SYNOPSIS The clinical and bacteriological findings in three cases of cryptococcal meningitis which occurred in Hong Kong are reported. The bacteriological investigations are discussed.

Littman and Zimmerman (1956) reported that there were over 300 cases of human cryptococcosis on record, yet compared with other systemic mycoses human cryptococcosis is still a rare clinical entity. Of all cases of human cryptococcosis reported, cryptococcal meningitis or meningoencephalitis is the most frequently encountered. The nervous symptoms and the ease with which the causal organism can be isolated from the cerebrospinal fluid enable prompt diagnosis. As far as we are aware there have been eight cases of cryptococcosis reported in China, and the scattered reports have been comprehensively reviewed by Cheng (1956). Three more cases in which the patients were also Chinese appeared in Malaya and Singapore (Pallis, 1949; Loh, 1951; Russell and Dean, 1957). Nevertheless, with a large population and only a dozen or so cases reported, it indicates that cryptococcosis amongst the Chinese race must be rare. In contrast to this belief, we have encountered three cases of cryptococcal meningitis in Hong Kong between December 1958 and December 1961. All the patients were Chinese and all died. The clinical records and the bacteriological investigations of these three cases are presented here to show that the incidence of cryptococcosis is probably higher than that found in the medical literature.

CASE REPORTS

CASE 1 A man aged 30 years, occupation unknown, was admitted to a private hospital in November 1958 complaining of frontal headache, diplopia, insomnia, and rapid loss of weight. Stained smears of cerebrospinal fluid examined at that stage showed acid-fast bacilli. He was treated for tuberculosis with streptomycin, P.A.S. and I.N.A.H. for 14 days but there was no significant improvement and the patient then developed signs of cerebellar dysfunctioning. He was transferred to the Queen Mary Hospital on 5 December 1958 for further investigation.

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On admission, he was found to be mentally confused and drowsy. His temperature was 100°F., blood pressure 160/100 mm. Hg, and pulse rate 64 per minute. Left facial palsy, nystagmus, and bilateral papilloedema were noted. Ventriculography showed evidence of incomplete posterior fossa block. Tuberculous meningitis with basal adhesion was suspected, but smears of cerebrospinal fluid showed numerous yeast-like cells, which were subsequently identified as *Cryptococcus neoformans* by culture. At operation basal arachnoiditis with adhesion was observed and the adhesion was separated to establish free passage of cerebrospinal fluid. A portion of the meninges removed for histological examination showed cryptococcosis. Tubercle bacilli were not found by smears, culture, or guinea-pig inoculation. The patient died eight days after admission. Permission for necropsy was refused.

CASE 2 A man aged 31 years, single and a tailor by profession, came to Hong Kong from Shanghai 10 years ago and was well until two weeks before admission when he began to experience attacks of vertigo and severe, persistent headache over the occipital and temporal regions. He had had no fever since the onset of the illness. His parents and 11 brothers and sisters were living with him but none of them had the same complaint.

The patient was admitted to the Nethersole Hospital on 18 January 1960. On admission, he was mentally clear, had no neck rigidity, and Kernig's sign was negative. The fundi showed bilateral papilloedema with many small haemorrhagic spots. There were no palpable lymph glands. Blood pressure was 130/90 mm. Hg and the pulse rate was 80 per minute.

Haemoglobin was 14 g./100 ml.; W.B.C.s 6,700/c. mm. (74% neutrophils and 26% lymphocytes); E.S.R. 4 mm./hr.; the Kahn test was negative.

Cerebrospinal fluid was at a pressure of 250 mm. The Queckenstedt test showed a quick rise and slow fall and Pandey's test was negative. Concentrations of sugar were 10 mg./100 ml. and of chloride 460 mg./100 ml. No cobweb formation or acid-fast bacilli were seen.

On the second day after admission and on four occasions subsequently *C. neoformans* was found in the cerebrospinal fluid either in smears or by culture.

Radiographs of the chest showed tuberculous infiltration in the right middle zone; the skull was normal.

The patient was treated with nystatin (500,000 units) and sulphadiazine (1 g.) given by mouth every four hours. Subjectively he felt better, but his condition suddenly deteriorated on the tenth day after admission and he died six days later.

The patient was treated with amphotericin B, 1 mg. per kg. daily, given intravenously. In spite of the treatment, which had been given for 10 days, he died on 3 January 1962. *Escherichia coli* was isolated from blood three days before death but *C. neoformans* was not found, showing that the fungus had not been disseminated into the blood stream. Permission for necropsy was not available.

The growth was very profuse on Sabouraud's glucose medium incubated at 37°C. Well-developed colonies about 2 to 5 mm. in diameter, which were heaped up, dome-shaped, creamy white and mucoid (Fig. 3), formed within 48 hours. Smears from these cultures showed encapsulated budding yeast-like cells not unlike those seen in direct smears, except that the capsules were narrower and not so well defined and that the cells were more regular in size. Upon further incubation, the colonies changed from

Strain	Growth at 37°C.	Indole Production	Reduction of Nitrate to Nitrite	Urea Hydrolysis	KNO ³ Assimilation	Carbon Assimilation from							Pathogenicity for Mice	
						Glucose	Galactose	Maltose	Mannite	Mannose	Sucrose	Lactose		Salicin
I (culture from case 1)	+	—	—	+	—	+	+	+	+	+	+	—	—	+
II (culture from case 2)	+	—	—	+	—	+	+	+	+	+	+	—	—	+
III (culture from case 3)	+	—	—	+	—	+	+	+	+	+	+	—	—	+

FIG. 1. *C. neoformans* from cerebrospinal fluid of case 2 mounted in lacto-phenol cotton blue. Capsules are clearly distinguishable. $\times 600$.

FIG. 2. *C. neoformans* from cerebrospinal fluid of case 2 in Indian-ink preparation. $\times 275$.

creamy white to brown and were drier. Mycelium was not formed even after prolonged incubation both at 37°C. and at room temperature, nor was it formed in corn meal-agar subcultures. Though the growth was slower at room temperature between 25 and 28°C. it was just as good as at 37°C. upon further incubation. Subsequent tests in broth cultures with an initial pH of 7.4 showed that the pH of the medium increased with time, reaching 8.0-8.3 after one week's incubation at 37°C. for cultures of all three strains.

Growth on fresh and heated blood agar media yielded poor growth. Only minute pinpoint colonies measuring less than 1 mm. in diameter developed in two or three days' time. The colonies were not appreciably bigger even after many days of incubation. No haemolysis was noticed from growth in fresh blood agar and haemodigestion was not seen in heated blood agar medium.

Biochemically, none of the three strains produced indole from peptone broth, or reduced nitrate to nitrite. They produced acid but no gas in fluid medium containing glucose and mannose in two days. No change was observed in maltose, mannite, sucrose, salicin, lactose, and galactose after 14 days' incubation at 37°C. For carbon assimilation and nitrogen assimilation tests, the methods of Lodder and Kreger van Rij (1952) were followed. In the latter potassium nitrate (Analar) was used. A negative control was always included by inoculating the cultures onto each batch of the basal medium. The

FIG. 3. Culture of *C. neoformans* from case 2 in Sabouraud's glucose agar incubated at 37°C. for 48 hours.

results showed that carbon was assimilated from glucose, mannose, maltose, mannite, sucrose, and galactose but not from lactose and salicin. Nitrogen was not assimilated from potassium nitrate by any of the three strains. All strains hydrolysed urea within 24 hours of incubation. The results of all the biochemical tests are set out in Table I.

Sensitivity tests *in vitro* against antifungal agents such as actidione, nystatin (Mycostatin), and amphotericin B (Fungizone) were carried out. Because amphotericin B was not available when strains I and II were isolated, their stock cultures were used for testing. The antifungal agents were incorporated in melted Sabouraud's glucose agar (pH 7.0) at 45°C., poured on plates, and hardened immediately in the refrigerator. The concentrations of each agent used were: actidione 24, 2.4, and 0.24 µg./ml.; nystatin 100, 10, and 1 unit/ml.; amphotericin B 10, 1.0 µg./ml. The inoculum was one loopful of a 24-hour culture of the test strain in glucose broth (pH 7.0) streaked all over the surface of the plate. Glucose agar plates without antibiotics were seeded for control. All plates were incubated at 37°C. for two days, and results were read with the aid of a dissection microscope. The results are presented in Table II. All the three strains were inhibited by amphotericin B within the range of inhibition *in vitro* studied elsewhere. Strains I and II were more resistant to actidione than strain III and all three were unusually resistant to nystatin. According to the information provided by the manufacturer the activity of nystatin decreases when a suspension is stored at 37°C. The incubation temperature in our test definitely affected the result. On the whole, strain III differed from the other two strains in response to these antifungal agents. It was more sensitive to actidione but was more resistant to nystatin and amphotericin B than the other two strains.

Benham (1956) has pointed out that the degree of invasiveness for mice is an important diagnostic characteristic for species of *Cryptococcus* and that only *C. neoformans* is pathogenic. All our strains were inoculated into white mice soon after isolation. For each isolate eight mice were inoculated, four receiving intraperitoneal and the other four intracerebral injections. The size of the inoculum per mouse

for intraperitoneal injection was 0.5 ml. of a 24-hour glucose broth culture and that for intracerebral injection 0.1 ml. of the same culture. The progress of the infection among the animals was very irregular, but on the whole those injected intraperitoneally died within two weeks of inoculation and those injected intracerebrally within 10 days. Necropsy findings in the mice were essentially the same for all three strains. The lungs were consolidated but the extent differed in each individual mouse. The brains were soft and congested or normal in appearance. A few of the animals showed a slightly enlarged spleen. The livers and kidneys were apparently normal in gross appearance. Those injected intraperitoneally often showed congested vessels of the abdominal wall and increase of peritoneal fluid, which was gelatinous. Histological study of sections of the organs, including the brains, showed numerous cryptococcal cells in the tissue spaces and the lumen of the vessels but the cellular reactions were mild in contrast to the overwhelming number of organisms present.

One locally bred and one Dunklin-Hartley strain of guinea-pig, weighing about 350 to 400 g., were inoculated with each of the strains of *C. neoformans* isolated. One millilitre of a 24-hour glucose broth culture was injected subcutaneously into the left groin of each animal. The Dunklin-Hartley guinea-pigs died within one month of inoculation, varying from 25 to 30 days for strains I, II, and III respectively, whereas the locally bred animals showed an inconsistent result. That inoculated with strain I died 40 days after inoculation, whilst those inoculated with strains II and III survived for more than two months. *Post-mortem* examination of the Dunklin-Hartley guinea-pigs thus infected showed large numbers of cryptococcal cells in the lungs and spleens microscopically, less so in the kidneys. There was little tissue reaction in the form of proliferation of the reticulo-endothelial cells and the brains and livers showed no change. Though the number of guinea-pigs used for our test was small, it is clear that variation between strains in susceptibility of guinea-pigs to infection is an important factor.

From the above findings the diagnosis of *Cryptococcus neoformans* of the three strains is justified, for all of them fulfilled the criteria laid down by Littman

TABLE II
END-POINT OF INHIBITION *IN VITRO* OF *C. NEOFORMANS* TO ANTIFUNGAL AGENTS

	Actidione (µg./ml.)	Nystatin (units/ml.)	Amphotericin B (µg./ml.)
Strain I	2.4	10	0.1
Strain II	2.4	10	0.1
Strain III	0.24	100	1.0
End-point according to reference	0.24 (Littman and Zimmerman, 1956)	1.5 (E. R. Squibb & Sons, technical leaflet, 1961)	0.03 to 0.6 (Seabury and Dascomb, 1960)

and Zimmerman (1956). We had no opportunity to examine serologically the patients' sera for capsular reacting antibody or agglutinating antibody. Also we had not tested the serological relationship of our strains by the above-mentioned reactions. However, guinea-pigs inoculated with living cultures of the three strains were skin-tested for allergic reactions with heat-killed cultures of homologous and heterologous strains. The test was carried out as follows:—

One Dunklin-Hartley guinea-pig was inoculated subcutaneously with 0.5 ml. of a 24-hour glucose broth culture of each of the three isolates. Three weeks later the animal was inoculated intradermally into separate areas with heat-killed cultures (60°C. for one hour) of the three strains. Similarly heat-killed cultures of *C. neoformans innocuous* and *Candida albicans* were injected into other areas as controls. Skin reactions appeared after two days not only on areas injected with the three strains of *C. neoformans* but also on those injected with *C. neoformans innocuous* and *Candida albicans*.

DISCUSSION

In all eight cases of cryptococcosis reported in China, there were symptoms of meningeal involvement before death and in seven of them *C. neoformans* had been isolated from the cerebrospinal fluid (Cheng, 1956). In our series, all three cases ended fatally after the development of meningeal symptoms. It appears that cryptococcal meningitis or meningoencephalitis is still the most frequent cause of death in this fungal infection. The course of the disease is variable.

C. neoformans is widely distributed in nature, for example, in milk (Carter and Young, 1950), in soil (Emmons, 1951), in various animals (Littman and Zimmerman, 1956), and in pigeon's nests (Kao and Schwarz, 1957). The organism is extremely resistant to dessication. According to Cheng (1956), dry powder of two strains of *C. neoformans* stored for one year and eight months was still alive upon transferring it to fresh cultural medium. He also found that dried sputum containing the organism gave growth upon inoculation to cultural medium a year afterwards. The high resistance to drying certainly has some significance in its epidemiology. Inhalation of dust from a contaminated source is the most probable mode of infection. However, there was no definite evidence to link the occupations of the patients reported here to these sources of infection. Emmons (see McCullough, Louria, Hilbish, Thomas, and Emmons, 1958) has pointed out that cryptococcosis was a non-contagious mycosis; that none of the other members of the big family of case 2 had the same complaint supports this point.

Clinically, cryptococcal meningitis and meningoencephalitis are usually mistaken for tuberculous meningitis. This is especially true in the Far East, where tuberculosis is still very common.

The presence of thickly encapsulated spherical, budding, yeast-like cells in the sediment of the centrifuged cerebrospinal fluid is suggestive of cryptococcosis. The capsule is best demonstrated by an Indian-ink preparation but a wet smear mounted in lacto-phenol cotton blue is also useful, because it digests the red blood cells and leucocytes leaving the cryptococci and their capsules intact. The spherical shape of the fungus is an important criterion, for it helps in the histological and cultural diagnosis of cryptococcus because most other yeast cells are egg-shaped or elliptical in outline. This point had been emphasized by Emmons (see McCullough *et al.*, 1958).

It is not difficult to culture *C. neoformans* from the cerebrospinal fluid, and Sabouraud's glucose agar is the medium of choice. Duplicate sets incubated at 37°C. and at room temperature might be set up in case other fungi are looked for. As a rule, *C. neoformans* grows much faster at 37°C. than at room temperature on primary isolation. The differentiation of *C. neoformans* from the saprophytic cryptococci has been fully discussed by Benham (1956). In view of the irregularity of the results in the sugar fermentation test, the more reliable carbon assimilation test should be used instead. Though the number of strains tested by us was small, our results were in full agreement with those of Benham. All our strains hydrolysed urea in Stuart's medium within 24 hours. Seeliger (1956) found that this test was useful to screen the genus *Cryptococcus* from the urease-negative *Candida*. It is, therefore, useful for primary identification of *Cryptococcus* isolated from mixed cultures. Nevertheless, animal inoculation is essential to discriminate *C. neoformans* from the saprophytic *Cryptococcus* species. The white mice, which are regularly susceptible to *C. neoformans* and succumb in a shorter period of time, are the animals of choice.

Actidione (Carton, 1952), nystatin (Brown and Hazen, 1957), and amphotericin B (Louria, Feder, and Emmons, 1957; Biddle and Koenig, 1958; Fitzpatrick, Rubin, and Poser 1958; Seabury and Dascomb, 1960) are all drugs which have been employed with some success in the treatment of cryptococcosis. Sensitivity tests of our strains *in vitro* showed that they were sensitive to these antifungal agents. In clinical practice, actidione and nystatin are disappointing in the treatment of cryptococcal meningitis, because they do not reach appreciable levels in the cerebrospinal fluid and central nervous system after intramuscular or intravenous injection (Littman and Zimmerman, 1956). In addition,

nystatin is fairly toxic when administered parentally. Amphotericin B appears to provide promising results if it is used early. Treatment of cryptococcosis with amphotericin B has been observed to be successful by many workers. Only case 3 in our series was treated with amphotericin B, but no clinical improvement was achieved owing to the advanced stage of the disease. The failure of amphotericin treatment in systematic mycosis has been discussed fully by Utz and Andriole (1960).

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