Mycopathologia (2010) 170:315–330
DOI 10.1007/s11046-010-9328-z

Pathogenesis of Pulmonary *Cryptococcus gattii* Infection: A Rat Model

Mark B. Krockenberger · Richard Malik · Popchai Ngamskulrungroj · Luciana Trilles · Patricia Escandon · Susan Dowd · Chris Allen · Uwe Himmelreich · Paul J. Canfield · Tania C. Sorrell · Wieland Meyer

Received: 10 September 2009 / Accepted: 25 May 2010 / Published online: 15 June 2010

Abstract A model of pulmonary cryptococcosis in immunocompetent rats was developed to better understand the virulence of *Cryptococcus gattii.* Six isolates were studied, representing four molecular genotypes (VGI-*MAT*α, VGIIa-*MAT*α, VGIIa-*MAT***a**, VGIIb-*MAT*α), obtained from Australia, Vancouver (Canada) and Colombia. These originated from human patients, a cat and the environment and were administered intratracheally (i.t.) or transthoracically into Fischer 344 or Wistar-Furth rats in doses varying from 10^4 to 10^7 colony-forming units (CFU) in 0.1 ml of saline. With the exception of animals given the VGIIa-*MAT***a** isolate, rats consistently became ill or died of progressive cryptococcal pneumonia following i.t. doses exceeding 10^7 CFU. Affected lungs increased in

The authors Mark B. Krockenberger and Richard Malik made an equal contribution to this work.

M. B. Krockenberger · P. J. Canfield
Faculty of Veterinary Science, Building B14, University of Sydney, Sydney, NSW 2006, Australia

R. Malik (✉)
Centre for Veterinary Education, Conference Centre, Building B22, The University of Sydney, Sydney, NSW 2006, Australia
e-mail: Richard.Malik@sydney.edu.au

P. Ngamskulrungroj · L. Trilles · P. Escandon · S. Dowd · C. Allen · W. Meyer
Molecular Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Millennium Institute, Western Clinical School, University of Sydney, Westmead, NSW 2145, Australia

P. Ngamskulrungroj
Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand

L. Trilles
Mycology Laboratory, Evandro Chagas Clinical Research Institute, Oswaldo Cruz Foundation. Av. Brazil, 4365, 21040-900 Rio de Janeiro, Brazil

P. Escandon
Grupo de Microbiología, Instituto Nacional de Salud, Bogotá, Colombia

U. Himmelreich
Max Planck Institute for Neurological Research, Köln 50931, Germany

T. C. Sorrell
Centre for Infectious Diseases and Microbiology, Westmead Millennium Institute, Western Clinical School, University of Sydney, Westmead, NSW 2145, Australia

Present Address:
C. Allen
Royal Children's Hospital, Herston Road, Herston, QLD 4006, Australia

weight up to tenfold and contained numerous circumscribed, gelatinous lesions. These became larger and more extensive, progressing from limited hilar and/or tracheal lesions, to virtually confluent gelatinous masses. Disease was localized to the lungs for at least 3–4 weeks, with dissemination to the brain occurring in some animals after day 29. The dose–response relationship was steep for two VGI isolates studied (human WM179, environmental WM276); doses up to 10^6 CFU i.t. did not produce lesions, while 10^7 or more yeast cells produced progressive pneumonia. Intratracheal inoculation of rats with *C. gattii* provides an excellent model of human pulmonary cryptococcosis in healthy hosts, mimicking natural infections. Disease produced by *C. gattii* in rats is distinct from that caused by *C. neoformans* in that infections are progressive and ultimately fatal.

Keywords Cryptococcus · Cryptococcosis · *Gattii* · Rat · Lung · Meningitis

Abbreviations

AIDS	Acquired immune deficiency syndrome
AFLP	Amplified fragment length polymorphism
BALT	Bronchus-associated lymphoid tissue
CNS	Central nervous system
CSF	Cerebrospinal fluid
CFU	Colony-forming units
CT	Computed tomography
H&E	Haematoxylin and eosin
HIV	Human immunodeficiency virus
i.t.	Intratracheal
MLST	Multi-locus sequence typing
MRI	Magnetic resonance imaging
NMR	Nuclear magnetic resonance
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
RFLP	Random fragment length polymorphisms
SDA	Sabouraud's dextrose agar

Introduction

Cryptococcosis is a potentially fatal disease of humans and animals with a worldwide distribution. It can be caused by the closely related species, *C. neoformans* (comprising varieties *grubii* and *neoformans*) and *C. gattii*, which together comprise the *Cryptococcus neoformans* species complex [1]. These species can be further divided into serotypes based on antigenic differences in their capsules (*C. neoformans var. grubii* [serotype A]; *C. neoformans* var. *neoformans* [serotype D]; *C. gattii* [serotypes B and C]) and further into eight major molecular types (VNI/AFLP1, VNII/AFLP1A, VNIII/AFLP3, VNIV/AFLP2 and VGI/AFLP4, VGII/AFLP6, VGIII/AFLP5, VGIV/AFLP7), based on PCR fingerprinting, *URA5* RFLP and AFLP analyses [2, 3]. These divisions have been helpful in discerning differences in epidemiology and patterns of disease.

As a rule, cryptococcosis occurs sporadically in healthy hosts. In Australia, such patients are infected equally by *C. neoformans* and *C. gattii*, although there are regional variations. The highest incidence occurs in the Northern Territory where there are approximately 120 new human cases/million of population/year, predominantly in indigenous individuals, and often with poor outcomes [4]. Even with appropriate therapy, patients may die or suffer substantial morbidity, including permanent neurologic sequelae (e.g. blindness, deafness and motor deficits).

C. gattii is of particular interest, as there is a proven association between one molecular type (VGI) and certain eucalyptus trees endemic to Australia [5]. The high prevalence of disease caused by the VGIIb genotype in Arnhem Land (the 'Top End' of the Northern Territory) [4] and parts of South Western Australia (Ellis, Heath, Saul, Krockenberger and Malik, unpublished observations) suggests that such strains may be more virulent and have the potential to spread into new environments. Since 1999, there has been a high and escalating incidence of VGII infections in Vancouver Island, Canada characterized by high environmental concentrations of potentially infective propagules (likely basidiospores) [6]. In the 1990s, such an event, but occurring on a smaller scale, accounted for the death of over 100 sheep on a single property in Western Australia. This outbreak was likewise referable to a VGII infection ([7]; Ellis pers. comm.).

Cryptococcosis is the most common cause of meningitis in companion animals, especially cats [8–10], and the second most common infectious disease of koalas, an Australian arboreal marsupial dependent on eucalyptus trees for food and shelter [11]. Studies in the koala have established the role of

nasal colonization, subclinical disease and reactivation in naturally occurring cryptococcosis [12, 13]. Further insights can be gained by studying molecular genetics, immunology and proteomics in relation to disease of man and animals. Such studies are contingent on establishing suitable experimental models.

Cryptococcosis has been studied extensively in mice. This species is highly susceptible to varieties of the *C. neoformans* species complex. Different genetic backgrounds are readily available and mice are inexpensive to buy and house. Furthermore, infection can be established via several routes—intranasal inhalation [14–16], intratracheally (i.t.) [17–19], intravenously [17, 19], intraperitoneally [14], intracerebrally [20] and into the footpad [21]. In contrast, rabbits are inherently resistant to cryptococcosis and inoculation of unusual sites (subcutis, testes, cerebrospinal fluid [CSF]) and/or immunosuppression (corticosteroids) are required to establish reproducible disease [1, 22].

Surprisingly, little work has been done using rats despite the natural occurrence of disease in this and related species [23]. Rat models offer a number of advantages: subjects are large enough to permit manipulations such as endotracheal intubation, bronchoalveolar lavage, serial venipuncture, CSF collection, radiography, computed tomography (CT) and magnetic resonance imaging (MRI). The normal host response to infection has been elucidated after i.t. inoculation using a well characterized *C. neoformans* var. *neoformans* strain [18, 24]: an effective host response was attributed to the establishment of cell-mediated immunity, with cytokine elaboration and antibody mediated phagocytosis [18, 24]. The critical importance of humoral immunity working in concert with cell-mediated immunity was illustrated in this model, where despite early dissemination to other sites (spleen, kidney, brain), systemic disease was transient and self-limiting.

The number of *C. gattii* virulence studies in mice and other even simpler host models (e.g. nematodes, caterpillars) has increased following the Vancouver outbreak [25–28]. In the present study, we used immunocompetent rats as a model of cryptococcosis due to *C. gattii*. Our two-fold aims were to develop and standardize the model and to elucidate the pathogenesis of this primary fungal pathogen.

Materials and Methods

C. gattii Strains

Details of the nine isolates used in this study are summarized in Table 1. All were maintained as frozen stock at −80°C and as lyophilized culture in the culture collection of the Molecular Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Australia. Isolates were serotyped using the Crypto check™ agglutination test (Iatron Labs, Japan).

Isolates were retrieved and subcultured on Sabouraud's dextrose agar (SDA). Prior to inoculation, cells were cultured in yeast nitrogen broth (Difco) containing 1% glucose, buffered at pH 7.0 with 0.345% w/v MOPS (Sigma Chemicals, St Louis, MO, USA) at 28–30°C for 18–20 h. Logarithmic to early stationary phase cryptococcal cells were washed three times and resuspended in Dulbecco's phosphate-buffered saline (PBS), pH 7.2. Counts were estimated in a haemocytometer and subsequently confirmed by performing colony counts after plating.

Experimental Procedures

C. gattii inoculation. The methods of Goldman et al. [18] were used, with minor modifications. Female Fischer 344 rats (130–200 g) and male Wistar–Furth rats (200–250 g) were obtained from the Animal Research Council, Perth, Western Australia. Two different anaesthetic methods were used: (1) Rats were anaesthetized with halothane (4–5%) in 100% oxygen administered initially via a plexiglass induction chamber, and, after they had lost their righting reflex, using a face mask connected to Bain system and a precision vaporizer. (2) Animals were anaesthetized with isoflurane (3–4%) and subsequently given ketamine (11 mg/kg subcutaneously) after losing the righting reflex and prior to application of the facemask; ketamine was administered to stabilize the depth of anaesthesia and thereby permit sufficient time for i.t. instillation of yeast cells.

In pilot experiments, 10^4–10^7 colony-forming units (CFU) of cryptococci in 0.1 ml PBS were injected transthoracically into the left lung at the midpoint between the cardiac impulse and the xiphisternum

Table 1 *C. gattii* isolates used in this study

Isolate	Code no.	Molecular type	Serotype	Mating type	Experiments	Origin
I	WM179	VGI	B	α	1	Human clinical isolate, Sydney, N.S.W., Australia
II	WM199 (TPO519 = Fiora)	VGI	B	α	1	Human clinical isolate, Adelaide, S.A., Australia
III	WM163 (GC-27)	VGI	B	α	1	Environmental isolate, Goldcoast, Qld, Australia
IV	WM198 (VPB571-015 = McBride)ϕ	VGIIb	B	α (fertile)	1	Feline clinical isolate, Sydney, N.S.W., Australia
V	WM276 (TCS/SC1)*	VGI	B	α (infertile)	1, 2, 3	Environmental isolate, Sydney, N.S.W., Australia
VI	WM0232 (CDCR-265)	VGIIa	B	α	6	Human clinical isolate, Major virulent type (VGIIa), Vancouver Island, Canada
VII	WM05349	VGIIa	B	**a**	6	Human clinical isolate, VGIIa, Colombia
VIII	WM0239 (CDCR-272)	VGIIb	B	α	6	Human clinical isolate, Minor virulent type (VGIIb), Vancouver Island, Canada
IX	WM05335	VGIIa	B	α	6	Human clinical isolate, MATα, Colombia

ϕ Kluger EK, Karaoglu HK, Krockenberger MB, Della Torre PK, Meyer W, Malik R. Recrudescent cryptococcosis, caused by *Cryptococcus gattii* (molecular type VGII), over a 13-year period in a Birman cat. *Med Mycol* 2006;**44**:561–566

* This isolate is currently being used in a whole genome sequencing project (http://www.bcgsc.ca/gc/cryptococcus)

using a 50 U insulin syringe (27-gauge) inserted to a depth of 5–8 mm.

In the case of i.t. injections, rats were positioned on their back, their mouth opened by retracting the tongue forward and away from the tabletop. Any pharyngeal contents were removed with forceps. Using an operating otoscope (Welch Allyn), the laryngeal opening was visualized, usually after gently dislodging the soft palate from the epiglottis using a plastic probe. In some experiments, the vocal folds were desensitized using 2% lidocaine spray. Either 0.1 ml India ink or a suspension of cryptococcal yeast cells (10^4–10^7 CFU in 0.1 ml PBS) was instilled either into the trachea (adult rats) or at the opening of the larynx (in smaller juvenile rats), using a 23-gauge blunted spinal needle, or a 10-cm 16-gauge plastic intravenous (i.v.) cannula, attached to a 1.0 ml syringe. Immediately following inoculation, 0.5 ml of air was injected forcibly through the needle or cannula to flush residual fluid and yeast cells out of the apparatus dead space. This dispersed the suspension deep into the lower respiratory tract. Occasionally this procedure was not well-tolerated, with approx. 5–10% of rats suffering fatal respiratory arrest, while half of the remainder stopped breathing, but could be resuscitated by administering 100% oxygen and commencing external chest compressions until breathing resumed. In early experiments, animals were given a single prophylactic dose of a broad spectrum antibiotic (0.1 ml; amoxicillin 140 mg/ml, clavulanate 35 mg/ml; Clavulox®) subcutaneously prior to recovery from anaesthesia.

Rats were housed in a PC2 facility, weighed weekly, fed commercial pellets and tap water ad libitum and kept in groups of 4–5. They were examined daily and those that developed neurological signs or severe respiratory distress were euthanized. The date of death or euthanasia was recorded.

All experiments were carried out according to the Australian National Health and Medical Research Council Guidelines and with the approval of the University of Sydney and/or Westmead Hospital Animal Ethics committees.

Organ weight and culture. Rats were euthanized by inhalation of 100% CO_2 or an overdose of pentobarbitone (200 mg/kg intraperitoneally) following induction of anaesthesia with halothane or isoflurane. Lungs, spleen and brain were dissected out for histopathologic examination, weighed using an electronic balance and

plated onto SDA after titration using a mortar and pestle.

Serum cryptococcal antigen determinations. Blood (1.0–3 ml) was obtained from anaesthetized rats by cardiac puncture under anaesthesia as a terminal procedure. Serum was separated and stored at −20°C. The reciprocal titre of cryptococcal antigen was determined in a representative number of sera using a commercial kit (Crypto-LA™; Wampole, NJ), following pronase pre-treatment of serum at 56°C and subsequent denaturation at 106°C.

Histology. For each rat, the most affected lung was used for culture. The contralateral lung was fixed in 10% neutral buffered formalin, processed and embedded in paraffin. Tissue sections (4–6 μm) were stained with haematoxylin and eosin (H&E), periodic acid Schiff (PAS) and mucicarmine. The presence or absence of yeast cells, appearance, location (intracellular versus extracellular), accompanying inflammatory response and the distribution of cryptococci within the tissues were recorded. The size of the capsule was measured using a calibrated eyepiece graticule.

Immunohistochemistry (IHC). A panel of monoclonal antibodies were used to determine the staining characteristics of different molecular types of *C. gattii* using methods described previously [29].

Experimental Design

Effectiveness of Different VG Isolates (and doses) in Establishing Pulmonary Cryptococcosis (Expt. 1). A pilot study was conducted to determine the pathogenicity of several *C. gattii* isolates and to select isolates for subsequent studies. First, WM179, the standard VGI strain, was chosen for a preliminary dose–response experiment. Twenty-two rats (a combination of Fischer and Wistar strains) were injected transthoracically using inoculums of various sizes and using small numbers of rats per group, as follows: 10^4, 10^5, 10^6, 6×10^6, 3×10^7 and 6×10^7 CFU using 4, 3, 3, 5, 4 and 3 rats/group, respectively. One rat was inoculated with 0.1 ml of sterile PBS as a control. Secondly, 5 different *C. gattii* isolates—4 VGI ([WM199 (TP0519), WM163 (GC-27), WM179, WM276 (TSC/SC1)] and 1 VGIIb [WM198 (McBride)]) were injected transthoracically into the lung using a total of 39 rats (10^7 CFU dose). Number of rats/group varied between isolates, as follows: 8, 8, 8, 11, 12 rats for WM179, WM199, WM163, WM276 and WM198, respectively. Animals were euthanized at various times according to their clinical status, for up to 55 days post-inoculation.

Intratracheal Inoculation Study (Expt. 2). To determine the effectiveness of i.t. inoculation, 10 and 5 male Fischer rats (9-week-old), were inoculated i.t. with 10^7 CFU of WM276 and WM198, respectively. Rats were monitored for up to 6 weeks.

Dose–response and Time-course Studies (Expt. 3). Sixty-five female Fischer rats were infected using different inoculum sizes of the same environmental VGI isolate [WM276], in order to study early pathogenesis of pulmonary cryptococcosis, at the microscopic and ultrastructural level. An environmental isolate was selected for these studies on the premise that strains from human or veterinary patients may have adapted during passage to become more virulent. Female rats (approx. 160 g) were used in these studies, in which 0, 10^4, 10^6 and 10^7 yeast cells were injected i.t. with 9, 21, 18 and 17 rats per group, respectively. Animals were observed and euthanized at day 0, 3, 7, 14, 26, 37, 43 and 62.

Comparison of Five C. gattii VGII Strains (Expt. 4). Twenty juvenile male Fischer rats, weighing 130–160 g each, were used in this experiment. Each rat was anaesthetized (using isoflurane and ketamine) and given 10^7 *C. gattii* CFU i.t. in 0.1 ml PBS. Because of the small size of these rats, injections were typically made into the laryngeal opening, rather than directly into the trachea. Isolates utilized included: (1) a clinical fertile VGIIa-*MAT*α strain from Vancouver Island (CDCR-265) (the major outbreak genotype); (2) a Colombian clinical VGIIa-*MAT***a** strain; (3) a clinical Vancouver Island VGIIb-*MAT*α strain (CDCR-272) (the minor outbreak genotype); (4) a Colombian environmental VGIIa-*MAT*α strain and (5) a fertile VGIIb-MATα strain (McBride); There were 5 groups of 4 rats, each inoculated with a given isolate. Rats within each group were not identified individually, but housed together in a single cage. One rat from each group was terminated at day 22 post-inoculation and day 29 post-inoculation, while the remaining rats were euthanized on day 58 unless they died earlier. Spontaneous death or euthanasia of animals somewhat altered this scheme.

Results

Effectiveness of Different VG Isolates in Establishing Pulmonary Cryptococcosis (Expt. 1)

Preliminary dose–response experiments were conducted using the standard VGI isolate (WM179) to establish the optimum inoculum to be utilized in subsequent studies. Cryptococcal pneumonia was established when more than 6×10^6 CFU were injected into the lung (Table 2). Therefore, a dose of 10^7 CFU was used for subsequent strain-variation experiments, which demonstrated that the environmental VGI isolate [WM276] and a veterinary VGIIb isolate [WM198] produced larger lesions than other isolates tested, and were thus most suited to producing reproducible disease. Following transthoracic injection, cryptococcomas with or without diffuse cryptococcal pneumonitis and empyema developed within 5–10 days.

Intratracheal Inoculation Study (Expt. 2)

WM276 and WM198 were chosen because lesions produced by these isolates were striking and easily monitored. The i.t. route was selected because cryptococcal basidiospores and desiccated yeast cells from natural environmental reservoirs are thought to enter the tracheobronchial tree to initiate infection in human patients [1]. The experiment with India ink injections demonstrated that the i.t. technique dispersed the 0.1 ml inoculum deep into the bronchial tree, reaching the caudal lung lobes, with preferential distribution to lobes on the right.

General and Clinical Findings

For the experiment using WM276, 8/10 male rats had evidence of progressive, locally extensive disease at necropsy. In the remaining two animals, only limited lesions were evident. Although most rats appeared

Table 2 Detailed results for Expt. 1

Isolate	Inoculum size (CFU)	No. rats	Days to death or euthanasia	Gross necropsy findings	Histopathologic findings*	Number of rats that had clinical signs
PBS control	0	1	32	Normal	0	Not known
WM179	10^4	4	32	Normal	1	Not known
WM179	10^5	3	32	Normal	1	Not known
WM179	10^6	3	7-32	Normal	1	Not known
WM179	$6x10^6$	5	22-55	Minimal lesions	2	Not known
WM179	$3x10^7$	4	15-55	Diffuse lesions	1	Not known
WM179	$6x10^7$	3	22-42	Diffuse lesions	2	Not known
WM198 (McBride)	10^7	12	5-20	Diffuse lesions	2	7
WM179	10^7	8	20-50	Minimal lesions	2	1
WM276 (TSC/SC1)	10^7	11	10-28	Diffuse lesions	2	7
WM199 (TPO519)	10^7	8	20-50	Minimal lesions	1	0
WM163 (GC-27)	10^7	8	20-50	Minimal lesions	1	0

Shaded area = data from the preliminary dose response study. Non-shaded area = data from the strain-variation studies. *Histopathology findings based on host inflammatory response graded on a scale described as (0) no evident host response; (1) a limited suppurative, lymphocytic or mixed inflammatory response; and (2) a granulomatous inflammatory response, including the presence of multinucleate giant cells

outwardly normal and continued to grow, one animal developed respiratory distress (with bloody nasal discharge) sufficient to require euthanasia, while another had severe lung involvement at necropsy. Similarly, 4/4 rats infected with WM198 had progressive and extensive lung lesions (one rat died immediately after inoculation under anaesthesia). One rat eventually developed asymmetrical neurological signs and succumbed; an interesting necropsy finding, seen subsequently in similarly affected animals (Expt. 4), was the presence of wood shavings in the oral cavity and oesophagus, likely reflecting a deranged appetite (pica). Numerous cryptococcal cells were recovered from both lung and brain of three rats (including the one which died) in late stages of the infection with the WM198 isolate.

Gross and Histologic Findings

Almost identical findings were observed with both strains. Gross lesions consisted of miliary, pale, gelatinous spheres (2–3 mm diameter) (Fig. 1a, b). In some cases, miliary lesions coalesced so that an entire lung lobe was affected. There was a tendency for lesions to be more extensive in the caudal portions of the lung lobes. Affected lungs were substantially heavier (2.5–6.2 g) than normal lungs (0.7–0.8 g), did not collapse during dissection, and sank when placed in formalin. Cryptococcomas effaced normal pulmonary parenchyma by compression and extension. Yeast cells could be detected in alveoli, interstitium, airways, trachea and the tracheobronchial lymph nodes, appearing to be exclusively extracellular. In rats inoculated with WM198 there was evidence of dissemination to other tissues including brain, meninges and spleen. Positive extra-pulmonary histologic lesions or cultures were not seen until day 29 post-inoculation. Identical findings were made in Expt. 4, conducted some years later. Thus, dissemination was a late event in disease pathogenesis and occurred only in the setting of extensive lung disease and high serum antigen titres (2,048 to 16,384).

Dose–Response and Time-Course Study (Expt. 3)

General and Gross Findings

Doses of 10^3 and 10^4 CFU of WM276 did not initiate detectable infection of the lungs, and yeast cells could not be detected in pulmonary parenchyma, hilar lymph nodes or airways at the time of necropsy (after day 0). On the other hand, inoculums of 10^6 or 10^7 CFU established localized pulmonary cryptococcosis. More consistent infection and more diffuse lung lesions were observed with 10^7 CFU inoculums (Table 3). In animals that developed disease, there was a clear trend for lesions to become larger the longer the period that had elapsed from inoculation to

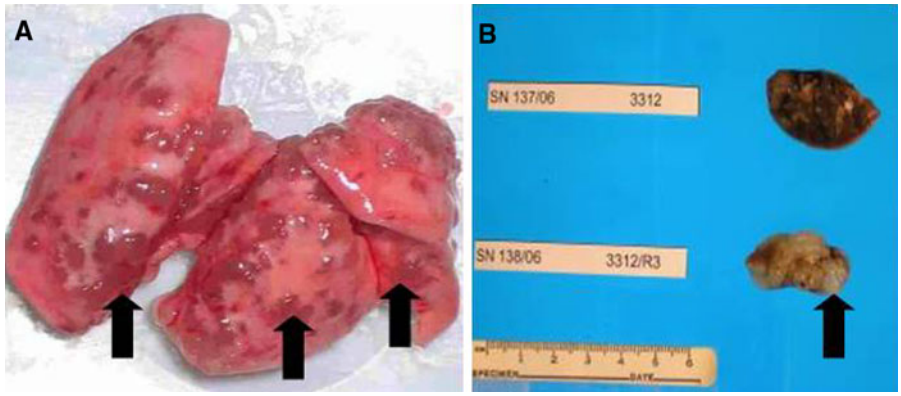

Fig. 1 Gross appearance of rat lungs in a Petri dish following dissection at necropsy (**a**). This rat was infected with a virulent fertile VGIIb isolate of the α-mating type WM198 (VPB571-015 = McBride). **b** Formalin-fixed lung illustrating variation in size of cryptococcomas in rats inoculated with WM276 (TCS/SC1). Cryptococcomas are evident as pale, gelatinous spheres (*arrows*)

Table 3 Findings of the dose–response and time-course study with WM276 (Expt. 3)

Inoculum size (CFU)	Number of infected rats/total rats	Dissemination to other organs
0	0/9	No
10^4	0/21	No
10^6	2/18	No
10^7	17/18	CNS (6), spleen (2) and kidney (1)

CNS central nervous system

* Numbers in bracket represents numbers of rats with disseminated disease

death or euthanasia. Lungs containing advanced lesions commonly weighed in excess of 2.0 g, and in some instances weighed in excess of 6.0 g.

Histologic Findings

Studying the pathogenesis of cryptococcosis over time, with reference to control animals inoculated with sterile PBS, the following features were noted. Rats inoculated with less than 10^6 CFU had no evidence of disease apart from non-specific early changes consisting of neutrophils and increased alveolar macrophages in the lung consistent with innate immunity. In rats inoculated with 10^6 CFU, animals occasionally developed detectable specific disease, although the majority recovered quickly and uneventfully. Animals inoculated with 10^7 CFU developed progressive disease, with a time course characterized by early innate immunity, development of acquired immunity, but with subsequent loss of an observable host response in the face of overwhelming infection. The comments set out below describe the features of an ultimately ineffective host response.

With i.t. doses of 10^6 and 10^7, up to two-thirds of cases developed tracheal inflammatory lesions (Fig. 2f) with numerous yeast cells present within the tracheal mucosa and occasionally submucosal tissues. In about half of the cases, tracheobronchial lymph nodes had identifiable microscopic lesions. With smaller inoculums, sporadic tracheal involvement was observed histologically. Dissemination was

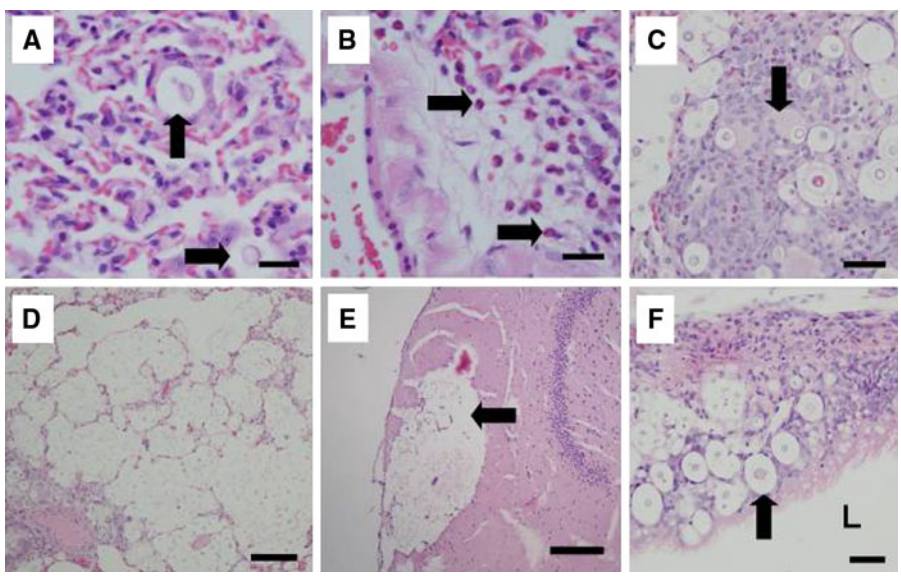

Fig. 2 Histologic findings of rats infected with *C. gattii*, WM276, with an inoculum size of 10^7 CFU. **a** Early changes in the lung following intratracheal inoculation. Low numbers of cryptococci (*arrows*) are evident within alveolar macrophages (day 2). **b** Perivascular eosinophils (*arrows*) are prominent in this section of lung (day 2). **c** Cryptococcal granuloma with multi-nucleate giant cell (*arrow*), epithelioid macrophages and eosinophils. **d** Representative microscopic appearance of a pulmonary cryptococcoma. Note the absence of a cellular host response. **e** Focal cerebral cryptococcoma (*arrow*). Note the absence of meningitis adjacent to the lesion. **f** Invasion of the tracheal mucosal epithelium by cryptococcal organism (*arrow*) (*Scale bars* represents 20, 20, 40, 100, 200, 40 μm for plates **a**, **b**, **c**, **d**, **e** and **f**, respectively. "L" represents tracheal lumen)

detected by positive culture and/or microscopy in approximately half of cases given 10^7 CFU.

Overall, there was a non-specific inflammation in the early period following inoculation. Subsequently, a more specific inflammatory response developed between days 2–12, with survival of a few cryptococci. Yeast cells began to replicate by day 12, with overwhelming multiplication of fungal cells by day 36. Eosinophils played a role in the host response, especially after 96 h. Yeast cells were minimally encapsulated and generally within phagocytes in the early stages. As disease progressed, cryptococcal cells were frequently observed outside phagocytes and had larger capsules. A chronology of the microscopic observations is given in Table 4.

Comparison of Five *C. gattii* VGII strains (Expt. 4)

The results from this experiment are presented in Table 5. Three rats died spontaneously during this experiment (at days 32, 38 and 56; Table 5); each of these rats had received a different isolate, and one demonstrated neurological signs immediately prior to death.

The interpretation of the data was complicated by the difficulty of reliably injecting the whole inoculum into the trachea of small juvenile rats. This problem probably accounted for the variable response of rats within a given treatment group.

The Colombian VGIIa-*MAT***a** isolate, despite originating from a human patient, was by far the

Table 4 Chronology of histological changes in a rat lung model of *C. gattii* strain WM276 (TSC/SC1) infection following inoculums of 10^7 CFU

Time	Inflammatory response	Cryptococcal cell characteristics
Day 1	Microscopic changes included moderate congestion and oedema, increased numbers of alveolar macrophages and neutrophils within alveolar walls and alveolar spaces. Approx. 50% of inflammatory cells were neutrophils, with the majority of the remainder being macrophages, with only a small component of eosinophils. Eosinophils were prominent in some areas, especially in connective tissue surrounding vessels. In some parts of lung, alveolar macrophages had engulfed cryptococci in the absence of neutrophils; in other areas, yeast cells were surrounded by neutrophils, with macrophages aggregated around the neutrophils. Inflammatory cells were unevenly distributed throughout the lung parenchyma	Few cryptococcal cells were present, most of which had minimal observable capsular material, in contrast to the occasional well-encapsulated organism. Yeast cells were generally quite small and most were associated with phagocytes. No budding of cryptococci was noted at this time point
Day 4	There was a marked pneumonia. Macrophages predominantly, and lesser numbers of neutrophils (5-10%) and eosinophils (about 10%), comprised the inflammatory infiltrate within alveolar walls and spaces. In some areas, macrophages were aggregating into sheets of epithelioid macrophages; but no multi-nucleate giant cells were evident in these small granulomas. The bronchus-associated lymphoid tissue (BALT) was more prominent than in control lung	Very few yeast cells were visible and most extracellular cryptococci had large capsules. Occasionally, smaller yeast cells with minimal capsules were evident intracellularly and in the midst of the small granulomas
Day 12	Small granulomas were present in the pulmonary parenchyma, as well as increased numbers of alveolar macrophages in alveolar spaces and increased prominence of the BALT. Neutrophils and eosinophils were sparse and patchy	Cryptococci were present in large airways as well as in pulmonary parenchyma. There were still very few yeast cells evident, although there was now evidence of replication (i.e. narrow-necked budding). Most extracellular yeast cells present had substantial capsules, whilst some smaller poorly encapsulated cells were present within phagocytes
Day 36	Only a limited macrophage response was evident. The BALT was of increased prominence. Very few neutrophils were present at this time; however, eosinophils were quite prominent, but with a patchy distribution	There were large aggregates of cryptococci within large airways and pulmonary parenchyma. A large proportion of yeast cells displayed budding. Most yeast cells present were large with prominent capsules

Table 5 Experiment 4

Data collected	Lung weight	Lung culture/ histology grade	Brain culture/ histology	Host response grade (lung)	Overall grade of disease/ *LCAT* & comments
(1) Vancouver Island VGIIa-MAT α	Human clinical isolate; major genotype seen in the Vancouver outbreak				
Day 22	0.9 g	++++/2	−/−	2	1
				IC	*LCAT* < 2
Day 29	1.5 g	++++/2	−/−	2	2
					LCAT 16
Day 58	0.9 g	++++/2	−/−	2	1
					LCAT 512
Day 58	8.6 g	++++/3	−/−	1	3
					LCAT > 8192
(2) Colombian VGIIa *MAT*a	Human clinical isolate				
Day 22	0.7 g	−/0	−/−	0	0
					LCAT 0
Day 29	0.7 g	++++/2	−/−	2	2
				IC	*LCAT* 32
Day 58	0.6 g	−/0	−/−	2	1
					LCAT 0
Day 58	0.8 g	−/0	−/−	2	*LCAT* 0
(3) Vancouver Island VGIIb-*MAT*α	Human clinical isolate; minor genotype seen in Vancouver outbreak				
Day 22	1.3 g	++++/3	−/−	2	2
				IC MNGC	
Day 29	0.7 g	+/0	−/−	0	0
					LCAT < 2
Day 32 (Rat died)	2.4 g	ND/3	ND/ Positive	1	3 Died; wood shavings in mouth and oesophagus
Day 58	1.3 g	++/0	−/−	2	1
					LCAT 32
(4) Colombian VGIIa *MAT*α	Environmental isolate				
Day 22	2.1 g	++++/2	−/−	2	2
				IC	
Day 29	0.9 g	++++/3	−/−	2	2
				IC MNGC	*LCAT*32
Day 38 (Rat died)	3.0 g	++++/3	++++/Positive	2 *IC*	Died; wood shavings in oesophagus; culture of spleen positive
Day 58	1.1 g	−/0	−/Equivocal	2	1
					LCAT 0
(5) McBride Australian VGIIb-*MAT*α	Veterinary isolate (cat with rhinosinusitis)				
Day 22	0.9 g	+++/1	−/−	1	1

Table 5 continued

Data collected	Lung weight	Lung culture/ histology grade	Brain culture/ histology	Host response grade (lung)	Overall grade of disease/ *LCAT* & comments
Day 29	2.5 g	++++/3	++	2	2 *LCAT* 4,096
Day 56 (Rat died)	6.2 g	++++/3	++++/Positive	1	3 Showed asymmetrical neurological signs immediately before death; wood shavings in the mouth and oesophagus
Day 58	3.6 g	++++/3	++++/Positive	2	3 No overt neurological signs *LCAT* > 8192

Data summarizing the main findings comparing the virulence of five *C. gattii* strains given intratracheally (10^7 CFU in 0.1 ml) to Fischer rats

ND not done, *LCAT* latex cryptococcal antigen agglutination titre, *IC* intracellular cryptococcal cells, *MNGC* multi-nucleate giant cells; Unaffected/control lung weights (same approx body weight) 0.7–0.9 g

Grades 0—no yeast cells/response; 1—mild; 2—moderate; 3—severe

least virulent strain in rats. Indeed, the absence of cultivatable yeast cells in rats necropsied at day 58 suggests that infections were self-limiting. In contrast, all four *MATα C. gattii* isolates were capable of producing progressive and extensive disease, with the development of multiple cryptococcomas throughout both lungs, resulting in encroachment upon and eventual obliteration of normal pulmonary tissue and either respiratory failure or late dissemination to the brain (and spleen). Such disease was associated with extremely high serum antigen titres, typically in excess of 512 (Table 5). The antigen titres of rats with macroscopic pulmonary lesions ranging from 16 to 16,384. All three animals which died had positive brain culture or histology at necropsy, suggesting they died a neurologic death. Although these rats had severe concurrent pulmonary involvement, two rats with lungs that were even heavier at necropsy did not succumb. One rat (VGIIb strain) showed depression and asymmetrical motor impairment shortly before dying; in contrast, another rat with culture and histologic meningitis was asymptomatic at the time of euthanasia.

Positive extra-pulmonary histologic lesions or cultures were not seen until day 32. Therefore, although dissemination could eventually occur, it was a late event and occurred only in the setting of extensive lung disease. In the rats with the smallest lesions, cryptococcal foci tended to be located near where bronchi entered the lung, close to the hilus.

Immunohistochemistry

The system used to differentiate between the relevant cryptococcal species in tissue sections utilized antibodies (all developed in Tom Kozell's laboratory) with known specificities viz. (1.) MAb471—positively labels capsule of all *Cryptococcus neoformans* species complex, (2.) mAb302—positively labels capsule of *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* and (3.) mAbF10F5—positively labels capsule of *C. neoformans* var. *grubii* and *C. gattii*.

By considering staining with all three antibodies, it is possible to differentiate *C. gattii* VGI, *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* from each other. Two limitations of this methodology were determined. First, no *C. gattii* specific antibody currently exists; therefore, the results of a staining panel must be considered and one must take into account negative staining. Secondly, VGII isolates have some cross reactivity with mAb302, and it is therefore impossible to distinguish between *C. gattii* VGII isolates and *C. neoformans* isolates in sections (Fig. 3).

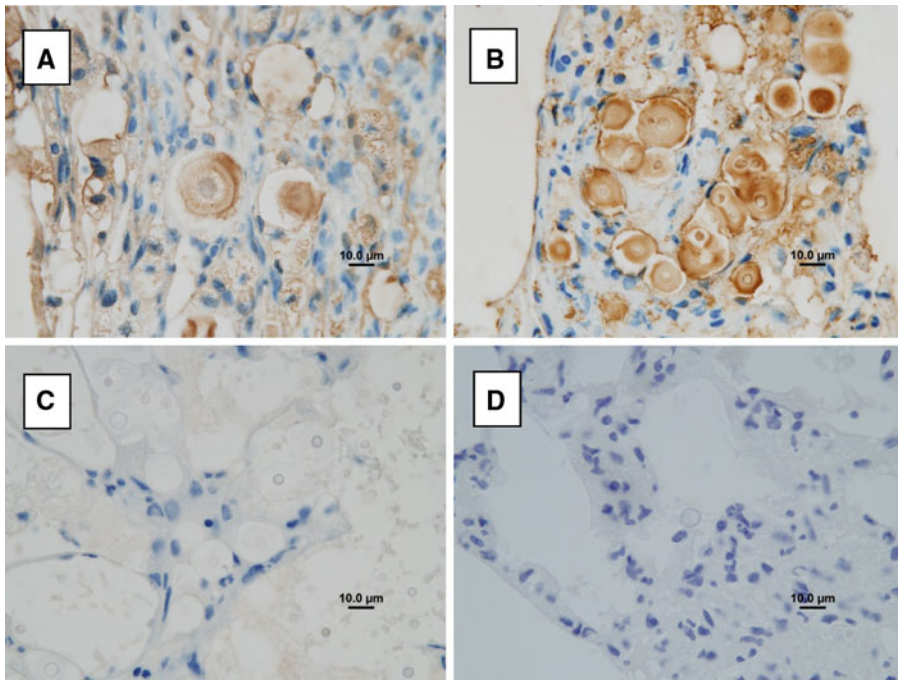

Fig. 3 Immunohistochemical staining technique. **a** Positive labelling using mAb471. **b** Positive labelling using mAbF10F5. **c** Negative labelling using mAb302. **d** Negative control. In all instances, the chromogen was DAB while Whitlock's' haematoxylin was the counter-stain. *Scale bar* 10 µm

Discussion

Adaptation of Goldman and Casadevall's rat model of pulmonary cryptococcosis to *C. gattii* resulted in a number of pertinent new findings. In terms of model standardization, although a cellular response was observed in rats infected with inoculums as small as 10^4 CFU, a dose of 10^7 CFU or higher was found to be most suitable for virulence and pathogenesis studies because it consistently produced gross lesions using either the transthoracic or i.t. route. It discriminated certain isolates (e.g. WM276, WM198, WM0232) from others of somewhat lower virulence (e.g. WM179, WM199, WM163 and especially WM05349) in Expt. 1 & 4. Even though the transthoracic injection technique was easier and faster to perform, the i.t. route was favoured because it simulates natural infection, where basidiospores are inhaled to cause primary infection of the lungs, with subsequently spread to other organs, e.g. meninges and brain [1].

Using this model, we discovered (1) the presence of tracheal lesions early in disease pathogenesis, (2) establishment of cryptococcal foci in tracheobronchial lymph nodes and (3) presence of limited peri-hilar lesions following i.t. dosing with marginal inoculums. A disadvantage of the i.t. route is that the number of yeast cells that reach the alveoli is somewhat unpredictable, as there are technical difficulties in ensuring that the whole dose is deposited with sufficient force to deeply penetrate the respiratory tract, especially in small rats.

A steep dose–response relationship was evident for *C. gattii* in the studies of VGI isolates WM179 and WM276 in Fischer rats, using both transthoracic (Expt. 1) and i.t. (Expt. 3) inoculation routes. Doses of 10^6 or less were presumably cleared before the infection could gain a foot-hold, likely as a result of physical defences such as the cough reflex, the mucociliary escalator and the innate cellular and chemical components of the inflammatory response. In contrast, doses in excess of 10^7 reliably produced lesions through the lungs, which once established, were incapable of being constrained. Eventually these infections disseminated haematogenously to the CNS. The difference between 10^6 and 10^7 CFU was crucial, a difference in one log making disease "all or

nothing". Such a critical dose–response relation for *C gattii* has not been reported previously. This may provide a reason why disease occurs sporadically in individual human patients (but not others) unlucky enough to receive heavy exposure from environmental reservoirs, particularly if the dose is deposited focally. This is in contrast to studies of murine models, in which dosing with smaller inocula simply results in a longer time period until the mice succumb.

In natural situations, where inhaled basidiospores would be first filtered by the nasal passages, with the lungs further protected by the cough reflex and the mucociliary blanket, it is likely that infection will begin as it did in our "technical failures", where a small number of cryptococcal cells took a toe-hold in a favourable location near the hilus, where main stem bronchi enter the lung. If lesions due to *C. gattii*, once established, are as relentlessly progressive as in our immunocompetent rats, one could envisage large cryptococcoma developing in this fashion.

Absence of effective phagocytosis in such lesions could account not only for lesion progression but also for absent early dissemination, as phagocytosis and intracellular survival may be critical for dissemination. The slow doubling time for *C. gattii* (2.4 days in vivo; data not shown) would, in part, account for the slowly progressive nature of lesions.

Cryptococcus gattii can cause persistent pneumonia in immunocompetent people similar to lesions caused by *Mycobacterium tuberculosis* [30]. Retrospective surveys of human patients have established that primary pulmonary cryptococcosis is often an indolent disease, with radiographic lesions being evident for months prior to symptoms developing [31]. Such disease may progress locally, or spread haematogenously to the CNS, or largely resolve, leaving residual foci in subpleural nodules or within hilar lymph nodes [31]. Of available animal models, the i.t. inoculated rat system provides the best facsimile for primary pulmonary cryptococcosis, encompassing the acute stage where infectious propagules first meet host defences, a later stage where yeast cells are successfully constrained in latent foci (*C. neoformans var. neoformans*) [18, 24] or result in progressive disease (*C. gattii*), and a reactivation phase produced by iatrogenic immunosuppression (*C. neoformans var. neoformans*).

This model of pulmonary cryptococcosis provides advantages over existing systems because: (1) rats are large and robust enough to perform a variety of inoculation procedures, (2) rats are tractable enough to permit investigations including pulse oximetry (using the tail) [32], transthoracic ultrasonography [33], whole body cross-sectional imaging using CT or MRI [34], repeated venipuncture [35], CSF collection [36] and bronchoalveolar lavage [37]; (3) several routes of drug administration are available; oral dosing with anti-infectives (using flavoured vehicles) is possible, furthermore drugs can be given by injection (e.g. interferon-δ, amphotericin B formulations) using subcutaneous, i.v. or intraperitoneal routes using physical restraint or light gaseous anaesthesia [8]. Combining these methodologies, it would be possible, for example, to determine whether subclinical disease could be cured using antifungal medication combined with adjunctive therapies, e.g. interferon or anticryptococcal antibodies.

Features of human pulmonary cryptococcosis were observed in the *C. gattii* rat model. In many patients with VGII infections, dissemination from the pulmonary focus occurs late in the clinical course, while in others disease is restricted to the lungs [5, 7]. Indeed, the pattern of disease in our model is close to the classic presentation in indigenous patients in the far north of Australia [4, 5]. These patients are often only examined late in the disease process, when they have extensive pulmonary disease, typically a solitary tumour-like lesion, or a small number of moderately sized necrotizing and/or fibrosing cryptococcomas with little inflammatory response [38]. Other examples of this presentation have been documented [31].

The key finding of the present work is that in two different strains of immune competent rat, numerous *C. gattii* strains produce progressive and ultimately fatal pneumonia, with late dissemination to the meninges as the terminal event. This stands in contradistinction to studies with the *C. neoformans var. neoformans* isolate (ATCC 24067) [18]. Using this strain, 200 g Fischer rats given 1.7×10^7 CFU (in 0.3 ml PBS i.t.) developed diffuse, subacute, self-limiting pneumonia, with early (day 7) low-level dissemination to brain, spleen and kidney. The limited extent of disease was reflected by low serum antigen titres (generally < 2). Signs of disease were absent, no rats died and the immune response largely cleared the infection, but with persistence of some yeast cells within macrophages and epithelioid cells. Even weaker virulence was shown by *C. gattii* VGIIa strain of the **a** mating type used in our

final series of experiments, where rats developed self-limiting disease, presumably due to an immune response which cleared the lung of yeast cells. The reduced virulence of **a** mating type isolates has been noted in mice models.

In contrast, *C. gattii* strains produced progressive and ultimately fatal disease in rats with high cryptococcal antigen titres in serum (often > 512 after 50 days). In one experiment, a rat died of asphyxia due to obstruction of the nasopharynx with a cryptococcoma, as is seen in other species [9]. In some rats injected transthoracically, pleurisy was present in addition to cryptococcal pneumonia, presumably due to seeding of the pleural space during the inoculation procedure. The consistency with which lung lesions developed made rats suitable for NMR studies [39].

WM276 (TSC/SC1) and WM198 (McBride) isolates deserve special mention, as both have been used widely in cryptococcosis models. Both have an extremely prominent capsule (data not shown), a feature apparent on initial isolation (from the environment and feline nasal cavity, respectively), during growth on solid media and in vivo in rats (lung, subcutis, meninges and brain). It has been suggested that *C. gattii* isolates are generally more virulent than *C. neoformans* isolates in murine inhalation models [26]. It could be that the elaborate capsule of *C. gattii* isolates hinders phagocytosis while promoting survival in phagocytes, two features facilitating disease progression. In relation to an i.t. model, a large capsule may inhibit mucociliary clearance and defeat innate lower respiratory tract defences. On the other hand, an elaborate capsule may hinder penetration of the blood brain barrier, which may in part explain why involvement of the CNS occurred so late, if at all, despite pronounced lung disease.

The immunohistochemistry data demonstrated that unlike VGI isolates, VGII isolates cannot be definitively differentiated from *C. neoformans* using the method developed previously [29]. Because of the rarity of VGII isolates in the Sydney region, a VGII isolate was not included in the initial validation of this method [29], and this study was the first experimental attempt to validate the method for VGII strains. The first indication of this oversight came with histologic investigations of animals infected in the Vancouver Island outbreak [40]. The method relies on antibodies raised against capsular epitopes. The discordant results for VGI versus VGII indicate there are antigenic differences in the capsule of these two molecular types.

This work has established a model for examining the relative virulence of *C. gattii* isolates. Expanding this model to include VGIII and *C. neoformans* var. *grubii* strains will provide additional useful information. Such a model is relevant to countries like Canada (18), Australia [5, 42], Papua New Guinea [43], Brazil [3], California (USA) [44] and Colombia [41] where *C. gattii* infections are quite common, including different parts of Australia where VGI and VGIIb are variably endemic, with an indigenous Aboriginal population apparently at greater risk through increased exposure and/or other epidemiologic predispositions. The rat model is not without flaws, however, and the technical difficulty of reliably injecting the entire inoculum into the trachea of a small animal needs to be overcome in order to improve the accuracy of dose-related data.

In summary, the principal finding is that in normal rats, most *C. gattii* strains produce progressive and ultimately fatal pneumonia, with late dissemination to the meninges leading to death. This is consistent with the notion that *C gattii* is a primary pathogen of immune competent hosts.

Acknowledgments The authors would like to thank Associate Professor David Ellis (Children, Youth and Women's Health Service, Adelaide, Australia) for providing strains [WM199 (TPO519 = Fiora) and WM163 (GC-27)], and Krystyna Maszewska for strain maintenance and culturing. The work was supported by a NH&MRC grant (ID 990738) to WM. Richard Malik was supported by the Valentine Charlton Bequest of the Centre of Veterinary Education.

Addendum Since the submission of this paper we have begun to use a nasal catheterisation technique to deposit cryptococcal organisms into the lower respiratory tract of rats in place of the intra-tracheal (i.t.) inoculation method described by Goldman and Casadevall. A urinary Tom cat catheter lubricated with sterile chloramphenicol eye ointment is threaded up the ventral nasal meatus of an anaesthetised rat for a distance of 5–8 mm until the catheter is wedged tightly (Fig. 4). The catheter is then grasped firmly using the thumb and first digit and held securely in the nostril, while another operator injects cryptococcal organism vigorously into the nasopharynx (and subsequently the trachea, bronchi and lungs) as described in the paper. This method is easier, quicker and requires a lesser depth of anaesthesia than the i.t. method. Preliminary work suggests it is a more reliable way of producing pulmonary lesions.

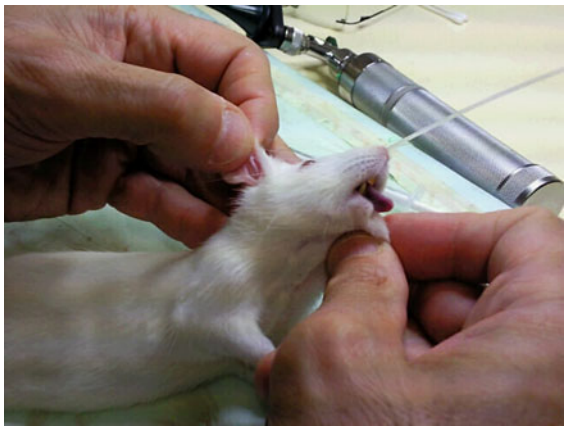

Fig. 4 An open ended urinary "Tom cat catheter" threaded up the ventral nasal meatus. The catheter is subsequently held with thumb and first digit where it enters the nostril, to ensure a leak proof seal prior to vigorous injection of a cryptococcal suspension into the nasopharynx

References

1. Casadevall A, Perfect JR. *Cryptococcus neoformans*. Washington, DC: ASM press; 1998.
2. Boekhout T, Theelen B, Diaz M, Fell JW, Hop WC, Abeln EC, et al. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. Microbiology. 2001;147:891–907.
3. Meyer W, Castaneda A, Jackson S, Huynh M, Castaneda E. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. Emerg Infect Dis. 2003;9:189–95.
4. Jenney A, Pandithage K, Fisher DA, Currie BJ. *Cryptococcus* infection in tropical Australia. J Clin Microbiol. 2004;42:3865–8.
5. Chen S, Sorrell T, Nimmo G, Speed B, Currie B, Ellis D, et al. Epidemiology and host- and variety-dependent characteristics of infection due to *Cryptococcus neoformans* in Australia and New Zealand. Australasian Cryptococcal Study Group. Clin Infect Dis. 2000;31:499–508.
6. Kidd SE, Hagen F, Tscharke RL, Huynh M, Bartlett KH, Fyfe M, et al. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). Proc Natl Acad Sci USA. 2004;101:17258–63.
7. Sorrell TC. *Cryptococcus neoformans* variety *gattii*. Med Mycol. 2001;39:155–68.
8. Malik R, Craig AJ, Wigney DI, Martin P, Love DN. Combination chemotherapy of canine and feline cryptococcosis using subcutaneously administered amphotericin B. Aust Vet J. 1996;73:124–8.
9. Malik R, Martin P, Wigney DI, Church DB, Bradley W, Bellenger CR, et al. Nasopharyngeal cryptococcosis. Aust Vet J. 1997;75:483–8.
10. O'Brien CR, Krockenberger MB, Wigney DI, Martin P, Malik R. Retrospective study of feline and canine cryptococcosis in Australia from 1981 to 2001: 195 cases. Med Mycol. 2004;42:449–60.
11. Krockenberger M, Stalder K, Malik R, Canfield P. Cryptococcosis in Australian Wildlife. Microbiol Aust. 2005;26(2):69–71.
12. Krockenberger MB, Canfield PJ, Barnes J, Vogelnest L, Connolly J, Ley C. et al. *Cryptococcus neoformans* var. *gattii* in the koala (*Phascolarctos cinereus*): serological evidence for subclinical cryptococcosis. Med Mycol. 2002;40:273–82.
13. Krockenberger MB, Canfield PJ, Malik R. *Cryptococcus neoformans* var. *gattii* in the koala (*Phascolarctos cinereus*): a review of 43 cases of cryptococcosis. Med Mycol. 2003;41:225–34.
14. Lim TS, Murphy JW, Cauley LK. Host-etiological agent interactions in intranasally and intraperitoneally induced Cryptococcosis in mice. Infect Immun. 1980;29:633–41.
15. Lima C, Vital JP. Olfactory mucosa response in guinea pigs following intranasal instillation with *Cryptococcus neoformans*. A histological and immunocytochemical study. Mycopathologia. 1994;126:65–73.
16. Riera CM, Masih DT, Rubinstein H, Nobile R, Vega VR. Effect of cyclophosphamide on rats experimentally infected with Cryptococcus neoformans. Mycopathologia. 1984;88:127–30.
17. Mody CH, Toews GB, Lipscomb MF. Cyclosporin A inhibits the growth of *Cryptococcus neoformans* in a murine model. Infect Immun. 1988;56:7–12.
18. Goldman D, Lee SC, Casadevall A. Pathogenesis of pulmonary *Cryptococcus neoformans* infection in the rat. Infect Immun. 1994;62:4755–61.
19. Kawakami K, Koguchi Y, Qureshi MH, Zhang T, Kinjo Y, Yara S, et al. Anti-CD11 b monoclonal antibody suppresses brain dissemination of *Cryptococcus neoformans* in mice. Microbiol Immunol. 2002;46:181–6.
20. Blasi E, Barluzzi R, Mazzolla R, Mosci P, Bistoni F. Experimental model of intracerebral infection with *Cryptococcus neoformans*: roles of phagocytes and opsonization. Infect Immun. 1992;60:3682–8.
21. Perceval AK. Experimental Cryptococcosis: hypersensitivity and immunity. J Pathol Bacteriol. 1965;89:645–55.
22. Perfect JR, Wright KA, Hobbs MM, Durack DT. Treatment of experimental cryptococcal meningitis and disseminated candidiasis with SCH39304. Antimicrob Agents Chemother. 1989;33:1735–40.
23. Scrimgeour EM, Purohit RG. Chronic pulmonary cryptococcosis in a *Rattus rattus* from Rabaul, Papua New Guinea. Trans R Soc Trop Med Hyg. 1984;78:827–8.
24. Goldman DL, Lee SC, Mednick AJ, Montella L, Casadevall A. Persistent *Cryptococcus neoformans* pulmonary infection in the rat is associated with intracellular parasitism, decreased inducible nitric oxide synthase expression, and altered antibody responsiveness to cryptococcal polysaccharide. Infect Immun. 2000;68:832–8.
25. Chaturvedi S, Ren P, Narasipura SD, Chaturvedi V. Selection of Optimal Host Strain for Molecular Pathogenesis Studies on *Cryptococcus gattii*. Mycopathologia. 2005;160:207–15.
26. Fraser JA, Giles SS, Wenink EC, Geunes-Boyer SG, Wright JR, Diezmann S, et al. Same-sex mating and the origin of the Vancouver Island Cryptococcus gattii outbreak. Nature. 2005;437:1360–4.
27. Narasipura SD, Chaturvedi V, Chaturvedi S. Characterization of *Cryptococcus neoformans* variety gattii *SOD2*

reveals distinct roles of the two superoxide dismutases in fungal biology and virulence. Mol Microbiol. 2005;55: 1782–800.
28. Hicks JK, Heitman J. Divergence of protein kinase A catalytic subunits in *Cryptococcus neoformans* and *Cryptococcus gattii* illustrates evolutionary reconfiguration of a signaling cascade. Eukaryot Cell. 2007;6:413–20.
29. Krockenberger MB, Canfield PJ, Kozel TR, Shinoda T, Ikeda R, Wigney DI, et al. An immunohistochemical method that differentiates *Cryptococcus neoformans* varieties and serotypes in formalin-fixed paraffin-embedded tissues. Med Mycol. 2001;39:523–33.
30. Ducati RG, Ruffino-Netto A, Basso LA, Santos DS. The resumption of consumption—a review on tuberculosis. Mem Inst Oswaldo Cruz. 2006;101:697–714.
31. Baker RD. The primary pulmonary lymph node complex of cryptococcosis. Am J Clin Pathol. 1976;65:83–92.
32. Decker MJ, Conrad KP, Strohl KP. Noninvasive oximetry in the rat. Biomed Instrum Technol. 1989;23:222–8.
33. Rao KG, Woodlief RM. Excessive right subdiaphragmatic fat: a potential diagnostic pitfall. Radiology. 1981;138:15–8.
34. Schuster DP, Kovacs A, Garbow J, Piwnica-Worms D. Recent advances in imaging the lungs of intact small animals. Am J Respir Cell Mol Biol. 2004;30:129–38.
35. Schirmer WJ, Schirmer JM, Townsend MC, Fry DE. Imidazole and indomethacin improve hepatic perfusion in sepsis. Circ Shock. 1987;21:253–9.
36. Sorrell TC, Wright LC, Malik R, Himmelreich U. Application of proton nuclear magnetic resonance spectroscopy to the study of *Cryptococcus* and cryptococcosis. FEMS Yeast Res. 2006;6:558–66.
37. Lee YS, Chen PW, Tsai PJ, Su SH, Liao PC. Proteomics analysis revealed changes in rat bronchoalveolar lavage fluid proteins associated with oil mist exposure. Proteomics. 2006;6:2236–50.
38. Fisher D, Burrow J, Lo D, Currie B. Cryptococcus neoformans in tropical northern Australia: predominantly variant gattii with good outcomes. Aust N Z J Med. 1993;23:678–82.
39. Himmelreich U, Allen C, Dowd S, Malik R, Shehan BP, Mountford C, et al. Identification of metabolites of importance in the pathogenesis of pulmonary cryptococcoma using nuclear magnetic resonance spectroscopy. Microbes Infect. 2003;5:285–90.
40. Lester SJ, Kowalewich NJ, Bartlett KH, Krockenberger MB, Fairfax TM, Malik R. Clinicopathologic features of an unusual outbreak of cryptococcosis in dogs, cats, ferrets, and a bird: 38 cases (January to July 2003). J Am Vet Med Assoc. 2004;225:1716–22.
41. Escandon P, Sanchez A, Martinez M, Meyer W, Castaneda E. Molecular epidemiology of clinical and environmental isolates of the *Cryptococcus neoformans* species complex reveals a high genetic diversity and the presence of the molecular type VGII mating type a in Colombia. FEMS Yeast Res. 2006;6:625–35.
42. McGill S, Malik R, Saul N, Beetson S, Secombe C, Robertson I, Irwin P. Cryptococcosis in domestic animals in Western Australia: a retrospective study from 1995–2006. Med Mycol. 2009;47:625–39.
43. Seaton RA, Verma N, Haraqii S, Wembrii JP, Warrel DA. Visual loss in immunocompetent patients with *Cryptococcus neoformans* var. *gattii* meningitis. Trans R Soc Trop Med Hyg. 1997;91:44–9.
44. Iqbal N, DeBess EE, Wohrle R, Sun B, Nett RJ, Ahlquist AM, Chiller T, Lockhart SR. *Cryptococcus gattii* Public Health Working Group. Correlation of genotype and in vitro susceptibilites of *Cryptococcus gattii* strains from the Pacific Northwest of the United States. J Clin Microbiol. 2010;48:539–44.