HIV-uninfected healthy patients. 第87回日本感染症学会、第61回日本化学療法学会総会合同学会、6月5日-6日、横浜、2013.

3）大久保陽一郎、大野秀明、篠崎　稔、宮﨑義継、根本哲生、若山　恵、栃木直文、石渡誉郎、中山晴雄、下平佳代子、安藝恭子、田辺公一、金子幸弘、梅山　隆、山越智、渋谷和俊. ガッティ型クリプトコックス症に関する感染防御機構ならびに病原因子の解析. 第57回日本医真菌学会総会・学術集会、9月27－28日、東京、2013.

4）田辺公一、大野秀明、金子幸弘、梅山隆、山越　智、名木　稔、知花博治、亀井克彦、宮﨑義継. 日本のキャンディン耐性カンジダの現状. 第57回日本医真菌学会総会・学術集会、9月27－28日、東京、2013.

5）大野秀明、大久保陽一郎、金子幸弘、田辺公一、梅山　隆、山越　智、亀井克彦、渋谷和俊、宮﨑義継. *Cryptococcus gattii* 感染書の病態解析（シンポジウム 4）. 第57回日本医真菌学会総会・学術集会、9月27－28日、東京、2013.

H. 知的財産権の出願・登録状況

（予定を含む。）

特許取得

なし

実用新案登録

なし

その他

特記事項なし

厚生労働科学研究費補助金（新型インフルエンザ等新興・再興感染症研究事業）
「地域流行型真菌症の疫学調査、診断治療法の開発に関する研究」班
分担研究報告書

*Cryptococcus gattii*の免疫原性に関する研究

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研究要旨　*Cryptococcus gattii*の病原性解明のため、その免疫原性についてin vivoおよびin vitroでの解析を行った。in vivoの解析では、CD4陽性細胞欠損による免疫抑制マウスを作製し、*C. gattii*を経気管的に感染させた結果、健常マウスに対して感染させた時と比較して有意に肺重量が減少したが、肺内生菌数に差異は認められなかった。菌の形態が免疫状態に応じて変化する可能性が示唆された。また、in vitroでの解析では、*C. gattii*から精製した莢膜多糖は*C. neoformans*と比較して樹状細胞の免疫応答を刺激しにくく、莢膜中の多糖組成も異なっており、莢膜多糖構造の違いによる低い免疫原性がin vivoでの高病原性に寄与している可能性が示唆された。

A．研究目的

*Cryptococcus neoformans*は、HIV感染症などの細胞性免疫不全者に感染しやすく、重篤化しやすいが、*Cryptococcus gattii*は、健常人での感染例も多く、宿主の免疫状態との関連性は不明である。*C. gattii*の病原性を知る上で、感染免疫に関する基礎的検討はきわめて重要である。

本研究では、*C. gattii*の病原性を免疫学的視点から解明することを目的として、in vivoおよびin vitroでの検討を行った。

B．研究方法

1. CD4陽性細胞欠損による免疫不全状態における*C. gattii*の病原性

モデル動物として、8週齢、メス、C57BL/6マウスを用い、菌株は*C. gattii* JP02株を用いた。健常マウスおよびCD4抗体によりCD4陽性細胞欠損状態としたマウスに経気管的に菌を接種し、15日間飼育後解剖し、肺重量と肺内生菌数を測定した。

2. in vitroでの免疫応答

C. neoformans H99株および*C. gattii* JP02株から莢膜多糖を抽出、精製し、C57BL/6の樹状細胞(JAWSII細胞)に接種して24時間培養後、上清中に産生されるIL-6量をELISA法で測定した。また、両株の莢膜多糖を陰イオン交換カラムクロマトグラフィーに供し、その溶出パターンを比較した。

C．研究結果

1. CD4陽性細胞欠損による免疫不全状態における*C. gattii*の病原性

健常マウスに対して感染させた時と比較して、CD4陽性細胞を欠損させることにより有意に肺重量が減少した（図 1）。また、肺内生菌数に差異は認められなかった。

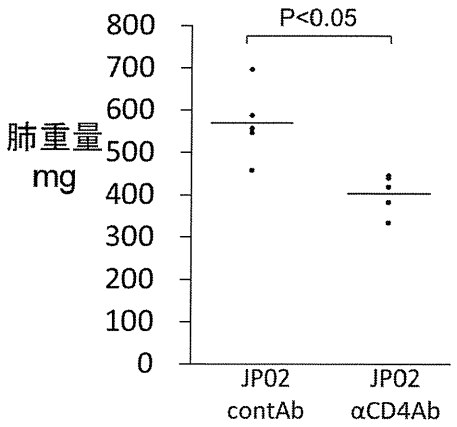

図 1　CD4 抗体投与の有無による肺重量の変化

2.　in vitro での免疫応答

H99 株から精製した莢膜多糖は濃度依存的に IL-6 の産生を誘導したが、JP02 株ではほとんど誘導しなかった（図 2）。また、両株の莢膜多糖を陰イオン交換カラムクロマトグラフィーに供した結果、その溶出パターンに顕著な違いが認められた。

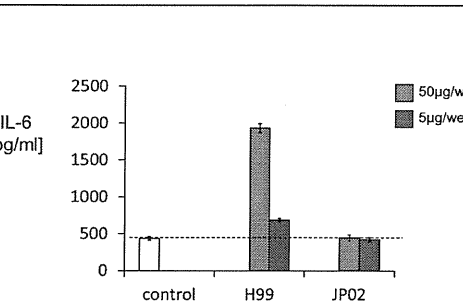

図 2　精製した莢膜多糖による樹状細胞からの IL-6 の誘導

D．考察

クリプトコックス症は、免疫不全者における発病が多く、また重篤化しやすいことが知られているが、*C. gattii* の免疫不全者における病原性については明らかにされていない。昨年度までに、CD4陽性細胞の欠損は *C. gattii* JP02 株感染よるマウス生存率に影響しないことを明らかにしたが、今回の検討において、JP02 株の形態が免疫状態に応じて変化する可能性が示唆された。

また、in vitro での解析では、JP02 株の莢膜多糖が H99 株と比較して樹状細胞の免疫応答を刺激しにくく、その莢膜中の多糖組成も異なっていたことから、JP02 株の莢膜多糖構造の違いによる低い免疫原性が in vivo での高病原性に寄与している可能性が示唆された。

E．結論

C. gattii の高病原性は、毒素産生といった宿主細胞への攻撃的なメカニズムではなく、*C. neoformans* とは異なる莢膜多糖構造による宿主免疫からの回避という防御的な因子が関与している可能性が示唆された。

F．健康危険情報

特記事項なし

G．研究発表

論文発表

英文

1　Kaneko Y, Miyagawa S, Takeda O, Hakariya M, Matsumoto S, Ohno H, Miyazaki Y. Real-time microscopic observation of Candida biofilm development and effects due to micafungin and fluconazole. Antimicrob Agents Chemother. 57:2226-2230, 2013.

2　Murakami Y, Fukasawa M, Kaneko Y, Suzuki T, Wakita T, Fukazawa H. Retinoids and rexinoids inhibit

hepatitis C virus independently of retinoid receptor signaling. Microbes Infect., 2013.

3 Okubo Y, Wakayama M, Ohno H, Yamamoto S, Tochigi N, Tanabe K, Kaneko Y, Yamagoe S, Umeyama T, Shinozaki M, Nemoto T, Nakayama H, Sasai D, Ishiwatari T, Shimodaira K, Yamamoto Y, Kamei K, Miyazaki Y, Shibuya K. Histopathological study of murine pulmonary cryptococcosis induced by Cryptococcus gattii and Cryptococcus neoformans. Jpn J Infect Dis. 66:216-221, 2013.

4 Migiyama Y, Kaneko Y, Yanagihara K, Morohoshi T, Morinaga Y, Nakamura S, Miyazaki T, Hasegawa H, Izumikawa K, Kakeya H, Hasegawa H, Kohno S. Efficacy of AiiM, an N-acylhomoserine lactonase, against Pseudomonas aeruginosa in a mouse model of acute pneumonia. Antimicrob Agents Chemother. 57:3653-3658, 2013.

5 Okubo Y, Tochigi N, Wakayama M, Shinozaki M, Nakayama H, Ishiwatari T, Shimodaira K, Nemoto T, Ohno H, Kaneko Y, Makimura K, Uchida K, Miyazaki Y, Yamaguchi H, Shibuya K. How Histopathology Can Contribute to an Understanding of Defense Mechanisms against Cryptococci. Mediators Inflamm. 2013:465319, 2013.

6 Ohno H, Tanabe K, Umeyama T, Kaneko Y, Yamagoe S, Miyazaki Y. Application of nested PCR for diagnosis of histoplasmosis. J Infect Chemother. 19:999-1003, 2013.

7 Kaneko Y, Fukazawa H, Ohno H, Miyazaki Y. Combinatory effect of fluconazole and FDA-approved drugs against Candida albicans. J Infect Chemother. 19:1141-1145, 2013.

和文

1 宮﨑義継, 田辺公一, 梅山 隆, 名木稔, 金子幸弘, 山越 智, 上野圭吾, 金城雄樹, 大川原明子, 大野秀明. アスペルギルス症. 感染症道場. 2:20-23, 2013.

2 大野秀明, 金子幸弘, 田辺公一, 梅山隆, 宮﨑義継. Cryptococcus gattii 感染症 -新興・再興感染症 up to date-. 化学療法の領域. 29 S-1:1144-1151, 2013.

3 金子幸弘, 浦井 誠, 宮﨑義継. カラーグラフィック連載「目で見る真菌と真菌症」 4. 治療薬の選択と投与. 化学療法の領域. 29:4-14, 2013.

4 浦井 誠, 金子幸弘, 宮﨑義継. ミニ特集 22 微生物の共存・共生と相互作用 ヒト vs 真菌 vs 細菌-人類の敵の敵は、味方か敵か-. 日本乳酸菌学会誌. 24:177, 2013.

学会発表

国際学会

1 Umeyama T, Yamagoe S, Tanabe K, Nagi M, Kaneko Y, Kinjo Y, Ohno H, Miyazaki Y. Mps1 kinase is required for normal nuclear segregation in Aspergillus fumigatus. 2013 Congress of Asia Pacific Society for Medical Mycology. June 19-23, 2013, Chengdu, China.

国内学会

1 金子幸弘, 宮﨑義継. リアルタイム観察によるカンジダバイオフィルムに対するミカファンギンの効果の解析. 第87回日本感染症学会学術講演会、第61回日本化学療法学会総会 . 6月 5-6 日, 2013年, 横浜.

2 井澤由衣奈, 大川原明子, 朴 貞玉, 金子幸弘, 川上和義, 竹山春子, 大石和徳, 金城雄樹. 肺炎球菌感染に対する蛋白・糖脂質併用ワクチンの防御効果. 第 24 回日本生体防御学会学術総会. 7月 10-12 日, 2013 年, 熊本.

3 梅山 隆, 山越 智, 田辺公一, 名木 稔, 金子幸弘, 金城雄樹, 大野秀明, 宮﨑義継. Aspergillus fumigatus の Mps1 キナーゼは正常な核の分裂に必要である. 第 7 回アスペルギルス研究会. 9 月 7 日, 2013 年, 東京.

4 梅山 隆, 山越 智, 田辺公一, 名木 稔, 金子幸弘, 金城雄樹, 大野秀明, 宮﨑義継. Aspergillus fumigatus の Polo-like キナーゼは菌糸伸長と分生子形成を制御している. 第57回日本医真菌学会総会・学術集会. 9 月 27-28 日, 2013 年, 東京.

5 浦井 誠, 金子幸弘, 田辺公一, 梅山隆, 大野秀明, 宮﨑義継. Candida albicans のアゾール感受性に対する併用薬の影響と作用機構に関する検討. 第57回日本医真菌学会総会・学術集会. 9 月 27-28 日, 2013 年, 東京.

6 田辺公一, 大野秀明, 金子幸弘, 梅山隆, 山越 智, 名木 稔, 知花博治, 亀井克彦, 宮﨑義継. 日本のキャンディン耐性カンジダの現状. 第57回日本医真菌学会総会・学術集会. 9 月 27-28 日, 2013 年, 東京.

7 大野秀明, 大久保陽一郎, 金子幸弘, 田辺公一, 梅山 隆, 山越 智, 亀井克彦, 渋谷和俊, 宮﨑義継. Cryptococcus gattii 感染書の病態解析（シンポジウム 4）. 第 57 回日本医真菌学会総会・学術集会. 9 月 27-28 日, 2013 年, 東京.

8 大久保陽一郎, 大野秀明, 篠崎 稔, 宮﨑義継, 根本哲生, 若山 恵, 栃木直文, 石渡誉郎, 中山晴雄, 下平佳代子, 安藝恭子, 田辺公一, 金子幸弘, 梅山 隆, 山越 智, 渋谷和俊. ガッティ型クリプトコックス症に関する感染防御機構ならびに病原因子の解析. 第57回日本医真菌学会総会・学術集会. 9 月 27-28 日, 2013 年, 東京.

9 金城雄樹, 金子幸弘, 朴 貞玉, 川上和義, 大石和徳, 宮﨑義継. 肺炎球菌蛋白・糖脂質併用ワクチンのマウスモデルによる評価. 第62回日本感染症学会東日本地方会学術集会・第 60 回日本化学療法学会東日本支部総会合同学会. 10 月 30-11 月 1 日, 2013 年, 東京.

H. 知的財産権の出願・登録状況
（予定を含む。）

特許取得
特記事項なし

実用新案登録
特記事項なし

その他
特記事項なし

III. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
秋山一男,大久保陽一郎, 太田利子, 久米田裕子, 坂元　仁, 渋谷和俊, 高鳥浩介, 高橋淳子, 田中真紀, 土戸哲明, 椿和文, 村松	感染症（章タイトル）	高鳥浩介,久米田裕子	カビのはなしミクロな隣人のサイエンス	朝倉書店	東京	2013	86-95
石野敬子	アミノグリコシド	高折修司	グッドマン・ギルマン薬理書［下］薬物治療の基礎と臨床［第 12 版］	廣川書店	東京	2013	1939-1960

雑誌

著者氏名	論文タイトル名	雑誌名	巻	ページ	出版年
Zhi Y, Sasai D, Okubo Y, Shinozaki M, Nakayama H, Yamagata Murayama S, Wakayama M, Ide T, Zhang Z, Shibuya K.	Comparison between the effectiveness of polymerase chain reaction and in situ hybridization in detecting the presence of pathogenic fungi by using the preserved DNA in formalin-fixed and paraffin-embedded tissues	Jpn J Infect Dis.	66	173-179	2013
Okubo Y, Wakayama M, Ohno H, Yamamoto S, Tochigi N, Tanabe K, Kaneko Y, Yamagoe S, Umeyama T, Shinozaki M, Nemoto T, Nakayama H, Sasai D, Ishiwatari T, Shimodaira K, Yamamoto Y, Kamei K, Miyazaki Y, Shibuya K.	Histopathological study of murine pulmonary cryptococcosis induced by *Cryptococcus gattii* and *Cryptococcus neoformans*.	Jpn J Infect Dis.	66	216-221	2013
Okubo Y, Ishiwatari T, Izumi H, Sato F, Aki K, Sasai D, Ando T, Shinozaki M, Natori K, Tochigi N, Wakayama M, Hata Y, Nakayama H, Nemoto T, Shibuya K.	Pathophysiological implication of reversed CT halo sign in invasive pulmonary mucormycosis: a rare case report.	Diagn Pathol.	8	82 (online journal)	2013
Oda M, Saraya T, Wakayama M, Shibuya K, Ogawa Y, Inui T, Yokoyama E, Inoue M, Shimoyamada H, Fujiwara M, Ota T, Takizawa H, Goto H.	Calcium oxalate crystal deposition in a patient with Aspergilloma due to *Aspergillus niger*.	J Thorac Dis.	5	E174-178	2013
Okubo Y, Tochigi N, Wakayama M, Shinozaki M, Nakayama H, Ishiwatari T, Shimodaira K, Nemoto T, Ohno H, Kaneko Y, Makimura K, Uchida K, Miyazaki Y, Yamaguchi H, Shibuya K.	How histopathology can contribute to an understanding of defense mechanisms against *cryptococci*.	Mediators Inflamm.	2013	465319 (online journal)	2013

Tochigi N, Okubo Y, Ando T, Wakayama M, Shinozaki M, Gocho K, Hata Y, Ishiwatari T, Nemoto T, Shibuya K.	Histopathological Imolications of *Aspergillus* Infection in Lung.	Mediators Inflamm.	2013	809798 (online journal)	2013
Ishiwatari T, Okubo Y, Tochigi N, Wakayama M, Nemoto T, Kobayashi J, Shinozaki M, Aki K, Sasai D, Yamamoto Y, Nakayama H, Shibuya K.	Remodeling of the pulmonary artery induced by metastatic gastric carcinoma: a histopathological analysis of 51 autopsy cases.	BMC Cancer.	14	14	in press
Ueno K, Okawara A, Yamagoe S, Naka T, Umeyama T, Utena-Abe Y, Tarumoto N, Niimi M, Ohno H, Doe M, Fujiwara N, Kinjo Y, Miyazaki Y.	The mannan of *Candida albicans* lacking β-1, 2-linked oligomannosides increases the production of inflammatory cytokines by dendritic cells.	Med Mycol.	51	385-395	2013
Ohno H, Tanabe K, Umeyama T, Kaneko Y, Yamagoe S, Miyazaki Y.	Application of nested PCR for diagnosis of histoplasmosis.	J Infect Chemother.	19	999-1003	2013
Kaneko Y, Miyagawa S, Takeda O, Hakariya M, Matsumoto S, Ohno H, Miyazaki Y.	Real-time microscopic observation of *Candida* biofilm development and effects due to micafungin and fluconazole.	Antimicrob Agents Chemother.	57	2226-2230	2013
Kaneko Y, Fukazawa H, Ohno H, Miyazaki Y.	Combinatory effect of fluconazole and FDA-approved drugs against *Candida albicans*.	J Infect Chemother.	19	1141-1145	2013
Norkaew T, Ohno H, Sriburee P, Tanabe K, Tharavichitkul P, Takarn P, Puengchan T, Burmrungsri S, Miyazaki Y.	Detection of environmental sources of *Histoplasma capsulatum* in Chiang Mai, Thailand by nested PCR.	Mycopathologia.	176	395-402	2013
金子幸弘, 浦井 誠, 宮崎義継.	カラーグラフィック連載「目で見る真菌と真菌症」4．治療薬の選択と投与	化学療法の領域	29	4-14	2013
町田安孝, 福島康次, 三好祐顕, 小原一記, 池田康紀, 亀井克彦, 宮崎義継, 福田 健.	経気管支鏡肺生検および気管支肺胞洗浄にて診断された慢性肺コクシジオイデス症の1例.	日本呼吸器学会雑誌	2	274-278	2013
宮崎義継, 田辺公一, 梅山 隆, 名木 稔, 金子幸弘, 山越 智, 上野圭吾, 金城雄樹, 大川原明子, 大野秀明.	アスペルギルス症.	感染症道場	2	20-23	2013

Miyasaka T, Akahori Y, Toyama M, Miyamura N, Ishii K, Saijo S, Iwakura Y, Kinjo Y, Miyazaki Y, Oishi K, Kawakami K.	Dectin-2-dependent NKT cell activation and serotype-specific antibody production in mice immunized with pneumococcal polysaccharide vaccine.	PLoS One.	8	e78611	2013
Tarumoto N, Kinjo Y, Kitano N, Sasai D, Ueno K, Okawara A, Izawa Y, Shinozaki M, Watarai H, Taniguchi M, Takeyama H, Maesaki S, Shibuya K, Miyazaki Y.	Exacerbation of Invasive *Candida albicans* Infection by Commensal Bacteria or a Glycolipid Through IFN-γ Produced in Part by iNKT Cells.	J Infect Dis.			in press
Nagi M, Tanabe K, Ueno K, Nakayama H, Aoyama T, Chibana H, Yamagoe S, Umeyama T, Oura T, Ohno H, Kajiwara S, Miyazaki Y.	The *Candida glabrata* sterol scavenging mechanism, mediated by the ATP-binding cassette transporter Aus1p, is regulated by iron limitation.	Mol Microbiol.	88	371-381	2013
大野秀明、金子幸弘、田辺公一、梅山 隆、宮崎義継.	*Cryptococcus gattii* 感染症.	化学療法の領域	29S-1	1144-1151	2013
大野秀明.	結核感染症の病態-結核発症の危険因子とは?-.	治療	95	1159-1163	2013
大野秀明、荒岡秀樹、梅山 隆、金子幸弘、宮崎義継.	接合菌症.	臨床検査	58	97-103	2013
Yamamoto Y, Izumikawa K, Morinaga Y, Nakamura S, Kurihara S, Imamura Y, Miyazaki T, Tsukamoto M, Kakeya H, Yanagihara K, Yasuoka A, Kohno S.	Prospective randomized comparison study of piperacillin/tazobactam and meropenem for healthcare-associated pneumonia in Japan.	J Infect Chemother.	19	291-298	2013
Kohno S, Izumikawa K, Yoshida M, Takesue Y, Oka S, Kamei K, Miyazaki Y, Yoshinari T, Kartsonis NA, Niki Y.	A double-blind comparative study of the safety and efficacy of caspofungin versus micafungin in the treatment of candidiasis and aspergillosis.	Eur J Clin Microbiol Infect Dis.	32	387-397	2013

Hosogaya N, Miyazaki T, Nagi M, Tanabe K, Minematsu A, Nagayoshi Y, Yamauchi S, Nakamura S, Imamura Y, Izumikawa K, Kakeya H, Yanagihara K, Miyazaki Y, Kugiyama K, Kohno S.	The heme-binding protein Dap1 links iron homeostasis to azole resistance via the P450 protein Erg11 in *Candida glabrata.*	FEMS Yeast Res.	13	411-421	2013
Nagaoka K, Yanagihara K, Harada Y, Yamada K, Migiyama Y, Morinaga Y, Hasegawa H, Izumikawa K, Kakeya H, Nishimura M, Kohno S.	Macrolides inhibit *Fusobacterium nucleatum*-induced MUC5AC production in human airway epithelial cells.	Antimicrob Agents Chemother.	57	1844-1849	2013
Mihara T, Izumikawa K, Kakeya H, Ngamskulrungroj P, Umeyama T, Takazono T, Tashiro M, Nakamura S, Imamura Y, Miyazaki T, Ohno H, Yamamoto Y, Yanagihara K, Miyazaki Y, Kohno S.	Multilocus sequence typing of *Cryptococcus neoformans* in non-HIV associated cryptococcosis in Nagasaki, Japan.	Med Mycol.	51	252-260	2013
Harada Y, Yanagihara K, Yamada K, Migiyama Y, Nagaoka K, Morinaga Y, Nakamura S, Imamura Y, Hasegawa H, Miyazaki T, Izumikawa K, Kakeya H, Kohno S.	In vivo efficacy of daptomycin against methicillin-resistant *Staphylococcus aureus* in a mouse model of hematogenous pulmonary infection.	Antimicrob Agents Chemother.	57	2841-2844	2013
Tanaka A, Nakamura S, Seki M, Fukudome K, Iwanaga N, Imamura Y, Miyazaki T, Izumikawa K, Kakeya H, Yanagihara K, Kohno S.	Toll-like receptor 4 agonistic antibody promotes innate immunity against severe pneumonia induced by coinfection with influenza virus and *Streptococcus pneumoniae.*	Clin Vaccine Immunol.	20	977-985	2013
Kaku N, Yanagihara K, Morinaga Y, Izumikawa K, Nagashima S, Kakeya H, Yamamoto Y, Fukuda M, Takatani H, Fukuda M, Hashiguchi K, Kohno S.	The definition of healthcare-associated pneumonia (HCAP) is insufficient for the medical environment in Japan: a comparison of HCAP and nursing and healthcare-associated pneumonia (NHCAP).	J Infect Chemother.	19	70-76	2013

Migiyama Y, Kaneko Y, Yanagihara K, Morohoshi T, Morinaga Y, Nakamura S, Miyazaki T, Hasegawa H, Izumikawa K, Kakeya H, Kohrogi H, Kohno S.	Efficacy of AiiM, an *N*-acylhomoserine lactonase, against *Pseudomonas aeruginosa* in a mouse model of acute pneumonia.	Antimicrob Agents Chemother.	57	3653-3658	2013
Yamada K, Yanagihara K, Kaku N, Harada Y, Migiyama Y, Nagaoka K, Morinaga Y, Nakamura S, Imamura Y, Miyazaki T, Izumikawa K, Kakeya H, Hasegawa H, Mikamo H, Kohno S.	Azithromycin attenuates lung inflammation in a mouse model of ventilator associated pneumonia by multidrug-resistant *Acinetobacter baumannii.*	Antimicrob Agents Chemother.	57	3883-3888	2013
Iwanaga N, Fukuda Y, Nakamura S, Imamura Y, Miyazaki T, Izumikawa K, Kakeya H, Yanagihara K, Soda H, Tashiro T, Kohno S.	ecrotizing pneumonia due to femoral osteomyelitis caused by community-acquired methicillin-resistant *Staphylococcus aureus.*	Intern Med.	52	1531-1536	2013
Tashiro T, Izumikawa K, Tashiro M, Morinaga Y, Nakamura S, Imamura Y, Miyazaki T, Kakeya H, Yamamoto Y, Yanagihara K, Hayashi T, Nagayasu T, Kohno S.	A case series of chronic necrotizing pulmonary aspergillosis and a new proposal.	Jpn J Infect Dis.	66	312-316	2013
Miyazaki T, Yamasaki N, Tsuchiya T, Matsumoto K, Hayashi H, Izumikawa K, Izumikawa K, Nagayasu T.	Partial lung resection of supernumerary tracheal bronchus combined with pulmonary artery sling in an adult: report of a case.	Gen Thorac Cardiovasc Surg.			in press
Takazono T, Izumikawa K, Suyama N, Kohno S.	Cavitary pulmonary metastases and aspergillosis: an autopsy case.	Intern Med.	52	1751-1752	2013

Yamada K, Yanagihara K, Kaku N, Harada Y, Migiyama Y, Nagaoka K, Morinaga Y, Nakamura S, Imamura Y, Miyazaki T, Izumikawa K, Kakeya H, Hasegawa H, Yasuoka A, Kohno S.	In vivo efficacy of biapenem with ME1071, a novel metallo-β-lactamase (MBL) inhibitor, in a murine model mimicking ventilator-associated pneumonia caused by MBL-producing *Pseudomonas aeruginosa.*	Int J Antimicrob Agents.	42	238-243	2013
Nagaoka K, Yanagihara K, Harada Y, Yamada K, Migiyama Y, Morinaga Y, Izumikawa K, Kakeya H, Nakashima M, Nishimura M, Kohno S.	Establishment of a new murine model of liver abscess due to *Fusobacterium necrophorum* via injection into caudal vein.	J Med Microbiol.	62	1755-1759	2013
Takahashi T, Maeda K, Suzuki T, Ishido A, Shigeoka T, Tominaga T, Kamei T, Honda M, Ninomiya D, Sakai T, Senba T, Kaneyuki S, Sakaguchi S, Satoh A, Hosokawa T, Kawabe Y, Kurihara S, Izumikawa K, Kohno S, Azuma T, Suemori K, Yasukawa M, Mizutani T, Omatsu T, Katayama Y, Miyahara M, Ijuin M, Doi K, Okuda M, Umeki K, Saito T, Fukushima K, Nakajima K, Yoshikawa T, Tani H, Fukushi S, Fukuma A, Ogata M, Shimojima M, Nakajima N, Nagata N, Katano H, Fukumoto H, Sato Y, Hasegawa H, Yamagishi T, Oishi K, Kurane I, Morikawa S, Saijo M.	The first identification and retrospective study of severe fever with thrombocytopenia syndrome in Japan.	J Infect Dis.			in press

Izumikawa K, Izumikawa KI, Takazono T, Kosai K, Morinaga Y, Nakamura S, Kurihara S, Imamura Y, Miyazaki T, Tsukamoto M, Yanagihara K, Hara K, Kohno S.	Clinical features, risk factors and treatment of fulminant *Mycoplasma pneumoniae* pneumonia: a review of the Japanese literature.	J Infect Chemother.			in press
Nagaoka K, Yanagihara K, Morinaga Y, Nakamura S, Harada T, Hasegawa H, Izumikawa K, Ishimatsu Y, Kakeya H, Nishimura M, Kohno S.	*Prevotella intermedia* induces severe bacteremic pneumococcal pneumonia in mice with up-regulated platelet-activating factor receptor.	Infect Immun.	82	587-593	2014
Morinaga Y, Yanagihara K, Gyotoku H, Oshima K, Izumikawa K, Yamasaki N, Kakeya H, Hayashi T, Fukuoka J, Nagayasu T, Kohno S.	Pulmonary artery pseudoaneurysm caused by *Streptococcus constellatus*.	Int J Infect Dis.	17	e1064-1066	2013
井上祐一、平山達朗、近藤 晃、泊 慎也、宮崎泰可、泉川公一、掛屋弘、山本善裕、栁原克紀、田代隆良、河野 茂.	肺炎球菌感染を併発したインフルエンザ肺炎に，脳梁膨大部病変を伴う脳症を合併した1成人例.	感染症学雑誌	87	451-456	2013
泉川公一、田代将人、河野 茂.	薬剤耐性アスペルギルスの現状、耐性獲得機序と今後.	日本化学療法学会雑誌	61	149-156	2013
Hashino S, Takahashi S, Morita R, Kanamori H, Onozawa M, Kawamura T, Kahata K, Kondo T, Tokimatsu I, Sugita T, Akizawa K, Asaka M.	Fungemia due to *Trichosporon dermatis* in a patient with refractory Burkitt's leukemia.	Blood Res.	48	154-156	2013
Sasai D, Okubo Y, Ishiwatari T, Sugita T, Kaneko T, Murayama SY, Shimamura T, Shinozaki M, Hasegawa C, Mitsuda A, Tochigi N, Wakayama M, Nemoto T, Shibuya K.	Histopathological evaluation of the efficacy of antifungals for experimental *Trichosporon* bloodstream infection.	Jpn J Infect Dis.	66	133-139	2013

Nakajima A, Saraya T, Mori T, Ikeda R, Sugita T, Watanabe T, Fujiwara M, Takizawa H, Goto H.	Familial summer-type hypersensitivity pneumonitis in Japan: two case reports and review of the literature.	BMC Res Notes.	6	371	2013
Zhang E, Tajima M, Tsuboi R, Sugita T.	Sequence diversity of the intergenic spacer region of the rRNA gene of *Cryptococcus albidus* isolated from the skin of patients with atopic dermatitis and healthy individuals.	Microbiol Immunol.	57	74-76	2013
Haga A, Tamoto H, Ishino M, Kimura E, Sugita T, Kinoshita K, Takahashi K, Shiro M, Koyama K.	Pyridone alkaloids from a marine-derived fungus, Stagonosporopsis cucurbitacearum, and their activities against azole-resistant *Candida albicans*.	J Nat Prod.	76	750-754	2013
Mekha N, Takashima M, Boon-Long J, Cho O Sugita T.	Three new basidiomycetous yeasts, *Pseudozyma alboarmeniaca*, sp. nov., *P. crassa* sp. nov., *P. siamensis* sp. nov isolated from Thai patients.	Microbiol Immunol.	58	9-14	2014
Yamamoto M, Takakura S, Hotta G, Matsumura Y, Matsushima A, Nagao M, Ito Y, Ichiyama S.	Clinical characteristics and risk factors of non-*Candida* fungaemia.	BMC Infect Dis.	13	247	2013

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Original Article

Histopathological Study of Murine Pulmonary Cryptococcosis Induced by *Cryptococcus gattii* and *Cryptococcus neoformans*

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SUMMARY: Although *Cryptococcus gattii* can cause life-threatening complications, putative virulence factors of *C. gattii* remain controversial. Therefore, we conducted the present study to elucidate the virulence factors of the yeast and found that the mortality rate of mice infected with *C. gattii* R265 was significantly higher than that of those infected with *C. gattii* 5815; however, no difference was found in the mortality rates between mice infected with *C. gattii* R265 and *Cryptococcus neoformans* H99. In contrast, we found a significant difference in histopathological findings of the lungs between mice infected with *C. gattii* R265 and *C. neoformans* H99. The former showed alveolar expansion due to yeast proliferation with much lesser macrophage response, whereas the latter showed numerous nodules in the alveolar space consisting of macrophages and multinucleated giant cells. Furthermore, alveolar expansion was more enhanced in mice infected with *C. gattii* R265 than in those infected with *C. gattii* 5815. Our study confirmed that there is a different pathophysiology leading to death during *C. gattii* and *C. neoformans* infections. The result can provide two characteristics of *C. gattii*: one includes some mechanisms to escape from host recognition via macrophage and another includes a high performance of pulmonary structural alteration. These characteristics may be associated with the high virulence of *C. gattii*.

INTRODUCTION

Cryptococcosis is an invasive fungal infection that can result following inhalation of *Cryptococcus neoformans* or *Cryptococcus gattii*. The latter species has recently emerged as a primary mammal pathogen on Vancouver Island, Canada (1) and has spread to various areas among animals and humans (2). Surprisingly, *C. gattii* can cause life-threatening infections in immunocompetent populations, whereas *C. neoformans* typically causes fatal diseases only in immunocompromised populations. Although the Vancouver Island outbreak emphasized the importance of understanding the pathophysiology of this emerging fungal pathogen, factors that define the virulence of *C. gattii* remain controversial. In the present study, we investigated and compared the biological characteristics of the pathogens and the pathophysiology of mice infected with different strains of cryptococci to elucidate the virulence factors of *C. gattii*.

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MATERIALS AND METHODS

Animals: Nine to 10-week-old female C57BL/6J mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were provided with sterile water and solid feed (FR-2; Funabasi Farm Co., Chiba, Japan). All animal studies were performed in accordance with the guidelines and permission of the animal experiment care committee of the National Institute of Infectious Diseases (NIID; Tokyo, Japan).

Microorganisms: *C. neoformans* H99 strain and *C. gattii* 5815 and R265 strains were used in this study. *C. neoformans* H99 was purchased from the American Type Culture Collection (ATCC; Manassas, Va., USA), *C. gattii* 5815 was clinically isolated and stored in the Medical Mycology Research Center of Chiba University, Chiba, Japan and kindly gifted to NIID, and *C. gattii* R265 was kindly provided by Dr. K. J. Kwon-Chung (National Institute of Health, Bethesda, Md., USA). The molecular types of *C. gattii* 5815 and R265 are VG I and VG IIa, respectively. These strains were stored at −80°C at NIID until use.

In vitro studies of pathogen biological characteristics:
(i) Growth rate at 37°C: All strains (*C. neoformans* H99, *C. gattii* 5815, and *C. gattii* R265) were preincubated with yeast peptone dextrose (YPD) broth at 30°C for 48 h. The fungal suspension was centrifuged, and the pellet was then washed once using fresh brain heart infusion (BHI) broth adjusted to pH 7.4. Fungal burden was adjusted to an optical density (OD) at 600 nm of 0.05, and the strains were then transferred to fresh BHI broth and cultured at 37°C. The OD at 660 nm of the culture was measured every hour for 7 days using a biophotorecorder TN-1506 (Advantec, Tokyo, Japan), and the doubling time at the exponential growth phase of each strain was calculated. The growth rate of each *Cryptococcus* strain was assessed by four independent evaluations.

(ii) Capsule thickness: To assess the capsule thickness of yeasts, the strains were preincubated in YPD broth at 30°C for 24 h. After preincubation, the yeast cells were pelleted and resuspended in Dulbecco's modified Eagle's (DME) medium (Invitrogen, Carlsbad, Calif., USA) and cell density was adjusted to 10^7 cells/ml using DME medium. The fungal cells were cultured at 37°C for 72 h, following which they were centrifuged, the supernatant was discarded, and the fungal pellet was resuspended in India ink. The capsule thickness of each cell was measured using an IX81 microscope (Olympus, Tokyo, Japan) and bio-imaging analysis software Lumina Vision (Mitani Corporation, Fukui, Japan). At least 100 cells were measured for each strain.

(iii) Melanin production: The strains were preincubated in BHI broth at 30°C for 24 h. After preincubation, the concentration of the cell suspension was adjusted to 0.5 at OD 600 nm, and serial 10-fold dilutions were prepared. Each fungal suspension was spotted onto bird seed agar (Becton, Dickinson and Co., Sparks, Md., USA) and incubated at 30°C and 37°C for 7 days. Finally, the extent of brown pigment formation was confirmed and assessed.

(iv) Proteinase activity: Proteinase activity of the fungal strains was determined on the basis of the report of Fotedar and Al-Hedaithy (3). In brief, the strains were preincubated with Sabouraud dextrose agar at 37°C for 48 h. After preincubation, the strains were suspended with phosphate buffered saline, and the fungal suspension was adjusted to 10^7 cells/ml using a cell counter. Following this, the cells were inoculated onto bovine serum albumin (BSA) agar (0.1% [w/v] BSA plus 0.01% [w/v] polypeptone) on three spots and cultured at 37°C for 7 days. Thereafter, proteinase activities of the yeast cells were measured by the observation of halo formation around the fungal colonies. *Candida albicans* ATCC90028 was used as a positive control.

(v) Phospholipase activity: Phospholipase activity of the fungal strains was also determined according to the methods described by Fotedar and Al-Hedaithy (3). The strains prepared as above were inoculated onto egg yolk agar medium instead of BSA agar and cultured at 37°C for 7 days. Thereafter, the phospholipase zone (Pz) value was determined by calculating the ratio of the diameter of the colony to the diameter of the colony and the precipitin zone. *C. albicans* ATCC90028 was used as a positive control. In the present study, the Pz value was classified as negative (Pz = 1.00), intermediate ($0.64 \leq$ Pz < 1.00), or positive (Pz < 0.64).

Infection: In total, 100 μl of fungal stock solution was inoculated into 10 ml of YPD broth and cultured for 48 h at 30°C with agitation. The culture suspension was centrifuged, and the fungal pellet was washed with sterilized saline once, centrifuged again, and the supernatant was discarded. The fungal pellet was resuspended in sterilized saline, and the suspension was adjusted to an OD of 0.5 at 600 nm, followed by 100-fold dilutions to prepare a working (inoculation) solution. The mice were anesthetized by spontaneous inhalation of isoflurane and then infected by intratracheal administration of 50 μl of fungal suspension (approximately $3.0–8.0 \times 10^3$ CFU/mouse). Subsequently, to evaluate the survival rate, six or seven mice were infected with each strain and observed daily for a period of 71 days, and three or four mice were infected for histopathological examination. The survival rate and histopathology were independently analyzed. Because mice infected with *C. gattii* R265 began to die 19 days after infection in our preliminary study, all the mice were sacrificed 14 days after infection to avoid variations in autopsy findings. The examination was performed twice on each *Cryptococcus* strain.

Preparation of histopathological specimens: The mice were euthanized through inhalation of carbon dioxide gas. The lung, liver, spleen, and brain of sacrificed mice were removed and fixed in 20% formalin for 48 h. After fixation, the organs were cut and embedded in paraffin wax. The paraffin-embedded organs were then cut into 4-μm-thick sections and stained with hematoxylin and eosin (HE) double stain, periodic acid Schiff (PAS) stain, and elastica stain for light microscopic observation.

Morphometric analysis of the lung structure: To determine the detailed level of structural alteration of the lung, we measured the cross point interval as referenced in previous investigations (4–6). The cross point interval is a useful indicator of alveolar size and variation, which is determined using the following procedure. Histopathological images of elastic-stained pulmonary sections were photographed using a video DP70 microscope camera (Olympus), and images were saved digitally. Parallel lines were overlaid onto each image using Image J software (version 1.46; National Institutes of Health, http://rsb.info.nih.gov/ij/). The distances along the line between the cross points indicated by the intersection of the line to the alveolar wall were then measured. All ambiguous structures, vascular structures, and lines touching the edges of the image fields were excluded from this analysis, and >2000 lengths in each group were measured.

Morphometric analyses of multinucleated giant cells (MGCs): To investigate the macrophage response, we measured the number of MGCs per unit area (mm^2), the number of nuclei per MGC, and the nuclear density within the cytoplasm, as referenced in previous investigations (7–9). MGC was defined as a cell containing at least two nuclei. The number of MGCs per unit area was determined by dividing the total number of MGCs observed in a pulmonary section by the area of the section. The number of nuclei per MGC was determined by counting the number of nuclei within MGCs. The nuclear density within the cytoplasm was calculated by

dividing the number of nuclei per MGC by the size of MGC. Histopathological images of pulmonary sections stained with PAS reaction were photographed using a BX 51 microscope (Olympus), and measurements were performed manually.

Statistical analysis: The time to mortality was evaluated for statistical significance using Kaplan-Meier survival curves, and *P*-values were obtained using the log-rank test. The doubling time of the yeasts, capsule thickness of the yeasts, cross point intervals, the number of nuclei per MGC, and nuclear density within the cytoplasm in each group were analyzed using one-way analysis of variance (ANOVA) and the Bonferroni post hoc test. Variances of the cross point intervals were analyzed using the *F*-test. *P*-value <0.05 was considered statistically significant.

RESULTS

Mortality of infected mice: On day 71 after infection, the cumulative mortality rates in mice infected with *C. neoformans* H99, *C. gattii* 5815, and *C. gattii* R265 were 7/7 (100.0%), 0/7 (0.0%), and 6/6 (100.0%), respectively (Fig. 1). According to Kaplan-Meier survival analysis with a log-rank significance test, the mortality rates of mice infected with *C. neoformans* H99 and *C. gattii* R265 were significantly higher than that of mice infected with *C. gattii* 5815, whereas no significant difference was found in the mortality rates between mice infected with *C. neoformans* H99 and *C. gattii* R265.

In vitro studies of pathogen biological characteristics: (i) Growth rate at 37°C: The doubling times (mean ± standard deviation [SD]) of *C. neoformans* H99, *C. gattii* 5815, and *C. gattii* R265 in BHI broth at 37°C were 3.66 ± 0.26, 6.28 ± 0.69, and 4.49 ± 0.12 h, respectively. ANOVA indicated that the doubling times of *C. neoformans* H99 and *C. gattii* R265 were significantly shorter than that of *C. gattii* 5815, whereas no significant difference was found in the doubling times between *C. neoformans* H99 and *C. gattii* R265.

(ii) Capsule thickness: The capsule thicknesses (mean ± SD) of *C. neoformans* H99, *C. gattii* 5815, and *C. gattii* R265 were 2.33 ± 1.05, 6.31 ± 1.55, and 3.22 ± 1.28 μm, respectively. ANOVA revealed significant differences for capsule thickness between all the groups.

(iii) Melanin production: Melanin production, which was assessed by verifying the extent of brown pigment formation, was confirmed in all the strains. Melanin production did not differ significantly among the strains.

(iv) Proteinase activity: No proteinase activity was detected in any of the strains.

(v) Phospholipase activity: No phospholipase activity was detected in any of the strains.

Histopathological examinations: (i) Conventional histopathological examination: Pulmonary sections of mice infected with *C. neoformans* H99 showed multiple well-demarcated nodules; however, the lung sections showed little expansion (Fig. 2A). Yeasts and numerous MGCs containing a large number of nuclei were observed only in these nodules (Fig. 2B and 3A). Pulmonary sections of mice infected with *C. gattii* 5815 showed relatively well-demarcated nodules and some

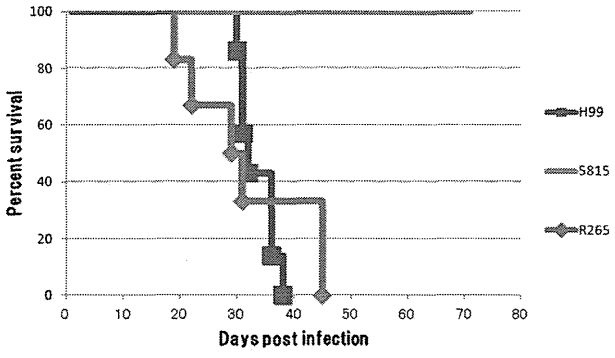

Fig. 1. On day 71 after infection, the cumulative mortality rates in mice infected with *Cryptococcus neoformans* H99, *C. gattii* 5815, and *C. gattii* R265 were 7/7 (100.0%), 0/7 (0.0%), and 6/6 (100.0%), respectively. Kaplan-Meier survival analysis with a log-rank significance test revealed that the mortality rates of mice infected with *C. neoformans* H99 and *C. gattii* R265 were significantly higher than that of mice infected with *C. gattii* 5815, whereas no significant difference was found in the mortality rates between mice infected with *C. neoformans* H99 and *C. gattii* R265. Differences were considered significant at $P < 0.05$.

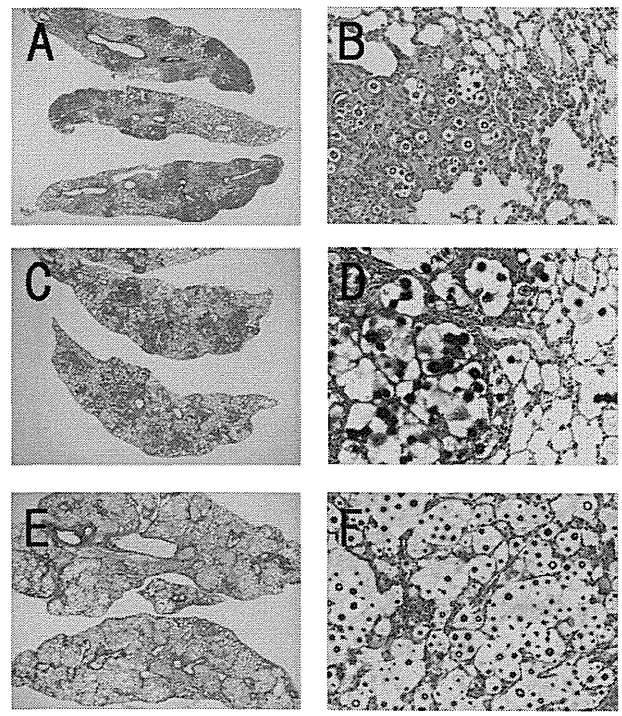

Fig. 2. (A) Pulmonary sections of mice infected with *Cryptococcus neoformans* H99 showed multiple well-demarcated nodular lesions (periodic acid Schiff [PAS] reaction, ×20). (B) Yeast cells were observed only in the nodular lesions, and alveolar airspace variation was not observed (PAS reaction, ×400). (C) Pulmonary sections of mice infected with *C. gattii* 5815 showed relatively well-demarcated nodular lesions and some degree of pulmonary enlargement (PAS reaction, ×20). (D) Yeasts were observed in these nodular lesions and surrounding alveoli. Some degree of alveolar airspace variation was observed (PAS reaction, ×400). (E) Pulmonary sections of mice infected with *C. gattii* R265 did not show nodular lesions but exhibited eccentric pulmonary enlargement (PAS reaction, ×20). (F) Numerous and irregular-sized yeast cells were diffusely observed in the alveoli. Marked alveolar airspace variation was also observed (PAS reaction, ×400).

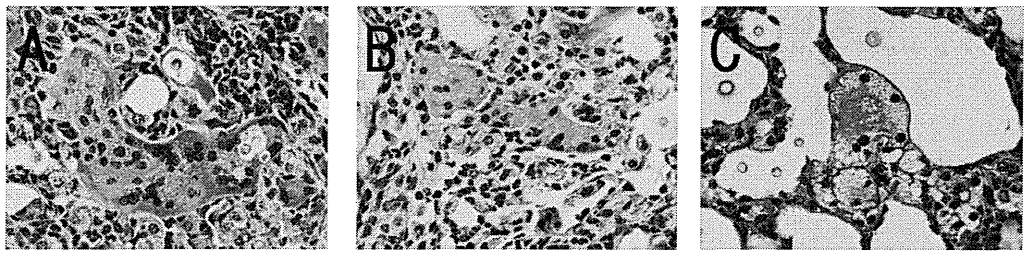

Fig. 3. (A) Pulmonary sections of mice infected with *Cryptococcus neoformans* H99 showed multinucleated giant cells (MGCs) with a well-developed and dense eosinophilic cytoplasm and a large number of nuclei. Most MGCs were of a foreign body type with less than 10 nuclei per cell (hematoxylin and eosin (HE) double stain, × 1000). (B) Pulmonary sections of mice infected with *C. gattii* 5815 showed MGCs with a somewhat foamy cytoplasm and a small number of nuclei that were loosely aggregated (HE double stain, × 1000). (C) Pulmonary sections of mice infected with *C. gattii* R265 showed MGCs with a foamy cytoplasm and a small number of nuclei that were loosely aggregated (HE double stain, × 1000).

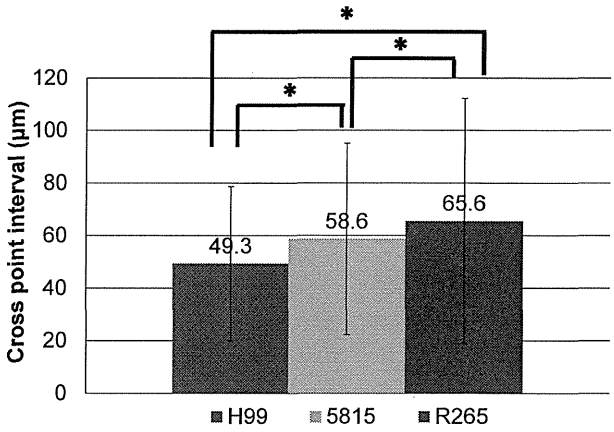

Fig. 4. The *F*-test revealed significant differences in the variances of the cross point intervals between all groups. One-way analysis of variance and Bonferroni post hoc test revealed significant differences in the mean value of cross point intervals between all groups. Differences were considered significant at $P < 0.05$ (*, $P < 0.05$).

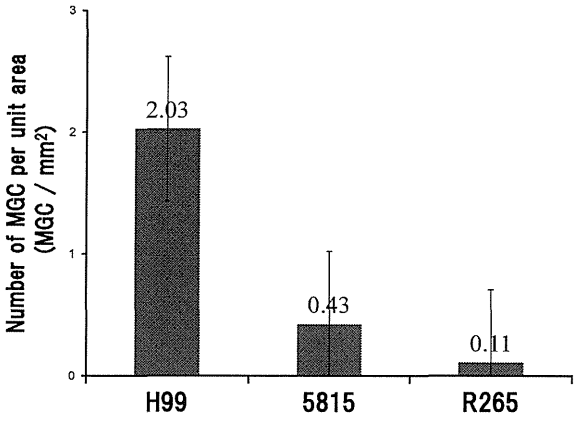

Fig. 5. Pulmonary sections of mice infected with *Cryptococcus neoformans* H99, *C. gattii* 5815, and *C. gattii* R265 showed that the numbers of multinucleated giant cells per unit area (mm^2, mean ± standard deviation) were 2.03 ± 0.38, 0.43 ± 0.26, and 0.11 ± 0.07, respectively. Values are expressed as mean ± standard deviation.

degree of pulmonary enlargement (Fig. 2C). Yeasts were observed in these nodules and surrounding alveoli. In addition, some degree of alveolar airspace variation was observed (Fig. 2D). MGCs were somewhat sparse and smaller than those observed in mice infected with *C. neoformans* H99 (Fig. 3B). Pulmonary sections of mice infected with *C. gattii* R265 did not show nodules, whereas eccentric enlargement of the alveolar space that contained numerous and irregular-sized yeasts was observed (Fig. 2E and 2F). In contrast, MGCs were extensively sparse and smaller than those observed in mice infected with *C. neoformans* H99 (Fig. 3C).

The brain, liver, and spleen were also examined in the present study; however, histopathological examination did not detect yeast cell proliferation in these organs.

(ii) Morphometric analysis of the lung structure: In pulmonary sections of mice infected with *C. neoformans* H99, *C. gattii* 5815, and *C. gattii* R265, the cross point intervals (mean ± SD) were 49.3 ± 29.4, 58.6 ± 36.5, and 65.6 ± 46.7 μm, respectively (Fig. 4). The *F*-test revealed significant differences in variances of the cross point intervals between all the groups. Furthermore, ANOVA revealed that all the three groups had significantly different cross point intervals (Fig. 4).

(iii) Morphometric analyses of MGCs: In pulmonary

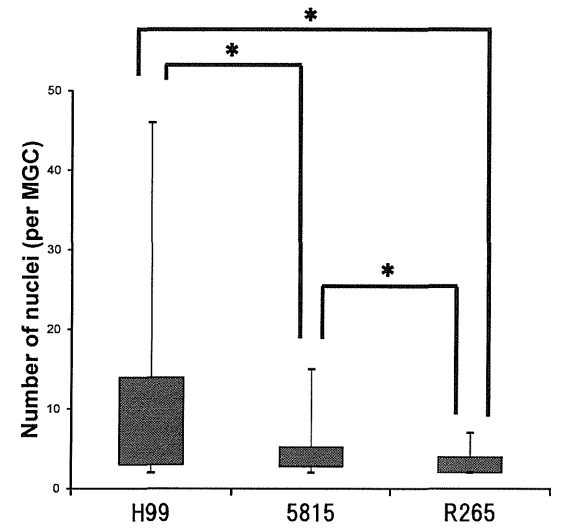

Fig. 6. The ends of the box represent the upper and lower quartiles of the number of nuclei per multinucleated giant cell (MGC). Bars at the end of the vertical lines are the data minimum and maximum values. One-way analysis of variance and Bonferroni post hoc test revealed significant differences in the mean number of nuclei per MGC between all groups. Differences were considered significant at $P < 0.05$ (*, $P < 0.05$).

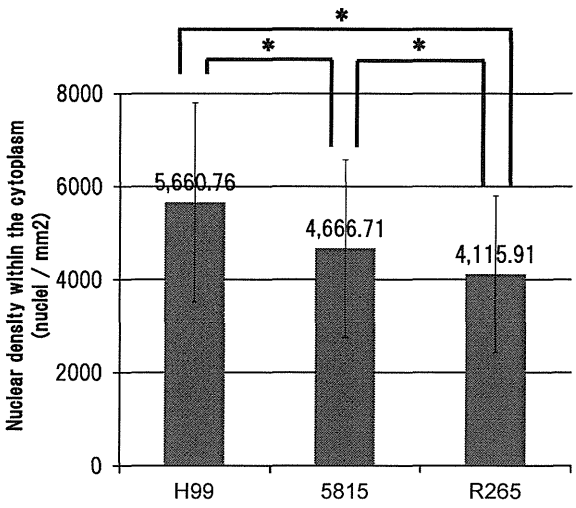

Fig. 7. The nuclear density within the cytoplasm of *Cryptococcus neoformans* H99, *C. gattii* 5815, and *C. gattii* R265 groups (nuclei/mm^2, mean ± standard deviation [SD]) was 5660.76 ± 2144.50, 4666.71 ± 1908.44, and 4115.91 ± 1683.13, respectively. One-way analysis of variance and Bonferroni post hoc test revealed significant differences in the nuclear density within the cytoplasm between all groups. Values are expressed as mean ± SD *, $P < 0.05$.

sections of mice infected with *C. neoformans* H99, *C. gattii* 5815, and *C. gattii* R265, the number of MGCs per unit area (mm^2, mean ± SD) was 2.03 ± 0.38, 0.43 ± 0.26, and 0.11 ± 0.07, respectively (Fig. 5). The number of nuclei per MGC (mean ± SD) in each group was 7.87 ± 6.03, 4.44 ± 2.53, and 2.87 ± 1.05, respectively. The nuclear density within the cytoplasm of each group (nuclei/mm^2, mean ± SD) was 5660.76 ± 2144.50, 4666.71 ± 1908.44, and 4115.91 ± 1683.13, respectively (see Fig. 7). ANOVA revealed significant differences in the number of nuclei per MGC and the nuclear density within the cytoplasm between all the groups (Fig. 6 and 7).

DISCUSSION

C. gattii can be divided into four discrete molecular types, VG I, VG II, VG III, and VG IV, which are estimated to have diverged from its sibling species, *C. neoformans*, approximately 37.5 million years ago (10). Unique clonal genotypes of the yeast are frequently associated with outbreaks (e.g., VG IIa, VG IIb, and VG IIc) (10). The most remarkable feature of *C. gattii* infection is its ability to cause life-threatening infections in healthy populations, as observed during the Vancouver Island outbreak. This outbreak also emphasized the importance of understanding the pathophysiology of this emerging fungal pathogen. Unfortunately, knowledge concerning the virulence factors of *C. gattii* in relation to the pathophysiology of the disease remains controversial. To elucidate the virulence factors of the yeast, we investigated the biological characteristics of pathogens and the pathophysiology of mice infected with three different strains of cryptococci. As a result, the mortality rates of mice infected with *C. gattii* R265 was significantly higher than that of mice infected with *C. gattii* 5815. However, there was no significant difference in the mortality rates between mice infected with *C. gattii* R265 and *C. neoformans* H99, although *C. neoformans* H99 typically causes fatal diseases only in immunocompromised populations. To gain a better understanding of the same high mortality rates between mice infected with *C. gattii* R265 and *C. neoformans* H99, we initially examined some biological characteristics, which have been regarded as cryptococcal virulence factors. The results showed that only the doubling time under physiological conditions (BHI agar adjusted to pH 7.4 at 37°C) suggested a correlation among the mortality rates of infected mice. Accordingly, the capability of rapid growth may be a candidate virulence factor of this yeast. Therefore, to gain insight into the pathophysiology more closely related to the virulence of cryptococci, we also conducted detailed histopathological examination and found significant differences in histopathological findings of the lung between mice infected with *C. gattii* R265 and *C. neoformans* H99. In particular, the former showed a very limited macrophage response but prominent eccentric enlargement of the alveolar space due to extensive proliferation of the yeast, while the latter showed an extensive macrophage response with giant cell formation. These results suggest that both extensive proliferation of the yeast in alveoli causing subsequent alveolar expansion during *C. gattii R265* infection and extensive macrophage response to invading *C. neoformans* H99 would significantly decrease pulmonary gas exchange properties of the alveolar septa. Consequently, it would be emphasized that the pathophysiology leading to death in *C. gattii* infection significantly differs from that in *C. neoformans* infection, although mice infected with both strains showed the same mortality rate.

Next, we discuss the virulence factors of *C. gattii*. In the present study, we found that the mortality rate of mice infected with *C. gattii* R265 was higher than that of mice infected with *C. gattii* 5815 and that the doubling time of *C. gattii* R265 was significantly shorter than that of *C. gattii* 5815. Histological findings induced by infection of both the strains were essentially similar. Lung sections from infected mice showed eccentric enlargement of the alveolar space, and the periphery of the enlarged alveoli was usually encompassed by collapsed septa lying on top of each other. This unique histological alteration was enhanced in mice infected with *C. gattii* R265 compared with those infected with *C. gattii* 5815, without change in quality. This finding was also confirmed by our cross point interval analysis, which is regarded as an indicator of structural alteration of the lung. These results suggest that alveoli invaded by inhaled cryptococci were expanded due to extensive yeast proliferation and that *C. gattii* R265 can cause greater structural alterations of the lung than *C. gattii* 5815. A part of the results may indicate that *C. gattii* R265 could reside and proliferate faster in the alveolar lumen than *C. gattii* 5815.

On the other hand, the poor macrophage response following infection by *C. gattii* strains is consistent with a previous report (11). A Th1-dominant cell-to-cell interaction triggered by the recognition of macrophages has been accepted as one of the most important defense mechanisms against cryptococcal infections (7). It has also been reported that cytoplasmic fusion of macrophages recruited at the foci of infection is accelerated by