Universidade Estadual de Londrina

LUCIENE AIRY NAGASHIMA

CARACTERIZAÇÃO PARCIAL DE FATORES HEMOLÍTICOS E CITOTÓXICOS DE *Arthrographis kalrae* E IMUNOMODULAÇÃO NO DECORRER DA INFECÇÃO EXPERIMENTAL MURINA

Londrina
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Tese apresentada ao Programa de Pós-graduação em Microbiologia, da Universidade Estadual de Londrina, como requisito à obtenção do título de Doutora.

Orientadora: Profa. Dra. Eiko Nakagawa Itano.

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NAGASHIMA, Luciene Airy. **Caracterização parcial de fatores hemolíticos e citotóxicos de *Arthrographis kalrae* e imunomodulação no decorrer da infecção experimental murina.** 2013. 66 p. Tese de Doutorado em Microbiologia – Universidade Estadual de Londrina, Londrina, 2013.

RESUMO

Arthrographis kalrae é um fungo dimórfico, neurotrópico e cosmopolita que vem sendo descrito como um raro patógeno humano com diversas manifestações clínicas. Este estudo investigou as atividades hemolítica e citotóxica de antígenos solúveis (*cell-free antigens* - CFA) de *A. kalrae* e a resposta imunopatológica ao fungo durante a infecção murina experimental. CFA total e suas frações cromatográficas (Sephadex G-150/120) foram testados em hemácias de camundongo para hemólise por teste em placa com leitura a 550 nm, e em linhagem celular P3U1 para citotoxicidade por método de metiltiazolil tetrazólio (MTT). Para caracterizar melhor o fator hemolítico, anticorpos anti-hemácias isogênicas sensibilizadas com CFA foram produzidos e testados em ensaio de inibição. Também, teste de hemólise foi realizado após aquecimento do CFA e a reatividade dos anticorpos foi analisada após tratamento com periodato. Na infecção experimental, camundongos Balb/c foram inoculados intravenosamente com *A. kalrae* ($5x10^6$ células) e a carga fúngica (unidades formadoras de colônias - CFU - e antigenemia por ELISA de inibição), análise histopatológica do cérebro, respostas imunes humoral (IgG e sub-classes IgG1, IgG2b e IgG2a, por ELISA) e celular (hipersensibilidade tipo tadio - DTH) e níveis de citocinas (IFN-γ, IL-4, IL-10 e IL-17) séricos e cerebrais foram avaliados 7, 14, 28 e 56 dias após infecção. Os resultados obtidos demonstraram atividades hemolítica e citotóxica em concentrações distintas e em frações de alta e baixa massa molecular (MM), respectivamente. Os anticorpos obtidos inibiram a hemólise, mas não a citotoxicidade. A atividade hemolítica não foi afetada pelo aquecimento e a sua maior reatividade foi detectada nas frações ricas em carboidratos, sendo essa atividade reduzida por tratamento com periodato. Em camundongos infectados foi detectada maior carga fúngica na fase inicial, principalmente no baço, fígado e cérebro, com pequena resposta inflamatória, com perda de peso e apresentando alterações comportamentais. No decorrer da infecção houve diminuição da carga fúngica, porém com CFU positivo em alguns órgãos mesmo após 56 dias de infecção.

Houve um aumento gradual no nível de IgG anti-*A. kalrae* (IgG1>IgG2a>IgG2b) com permanência alta de antigenemia e de menor peso corporal no decorrer de infecção, em relação ao grupo controle. Foi detectado aumento de resposta de DTH em 14 dias, ocorrendo diminuição após esse período. Houve diminuição de IFN-γ (14 a 56 dias), aumento de IL-4 (7 e 56 dias) e aumento (7 dias) ou diminuição (56 dias) de IL-17 no soro. Diminuição nos níveis das citocinas foi observada no cérebro 56 dias após a infecção. Concluímos que *A. kalrae* secreta fatores solúveis com atividade hemolítica e citotóxica, sendo o primeiro de natureza glicoproteica, termoestável e de alta MM, possivelmente distinto do fator citotóxico. Concluímos também que o isolado *A. kalrae* induz alteração comportamental, perda de peso, imunodepressão celular com modulação sistêmica de citocinas, possivelmente para padrão de resposta Th2, induzindo também modulação de citocinas no cérebro na infecção experimental em camundongos.

Palavras-chave: hemólise, citotoxicidade, resposta imune, citocinas, infecção fúngica.

NAGASHIMA, Luciene Airy. **Partial characterization of hemolytic and cytotoxic factors of *Arthrographis kalrae* and immunomodulation over the couse of the murine experimental infection.** 2013. 66 p. Thesis in Microbiology – State University of Londrina, Londrina, 2013.

ABSTRACT

Arthrographis kalrae is a dimorphic, cosmopolitan and neurotropic fungus that has been described as a rare human pathogen with several clinical manifestations. This study investigated the hemolytic and cytotoxic activities in *A. kalrae* cell-free antigens (CFA) and the immunopathological response to the fungus during murine experimental infection. Total CFA and its chromatography fractions (Sephadex G-150/120) were tested on mouse erythrocytes for hemolysis in plate assay by absorbance at 550 nm and on a P3U1 cell line for cytotoxicity by methylthiazolyl tetrazolium (MTT) method. In order to characterize hemolytic factor, antibodies against isogenic erythrocytes sensitized with CFA (anti-E-CFA) were produced and tested in inhibition assay. Additionally, hemolytic assay was performed after CFA heating and antibody reactivity of the chromatography fractions was analyzed after periodate treatment. In experimental infection, Balb/c mice were challenged intravenously with *A. kalrae* ($5x10^6$ cells) and the fungal load (colony-forming units – CFU - or antigenemia by inhibition-ELISA), histopathological analysis of the brain, humoral (IgG and IgG1, IgG2a and IgG2b subclasses, by ELISA) and cellular (delayed-type hypersensitivity - DTH) immune responses and levels of systemic and cerebral cytokines (IFN-γ, IL-4, IL-10 and IL-17) were evaluated 7, 14, 28 and 56 days after infection. Results showed hemolytic and cytotoxic activities in different concentrations and in high and low molecular mass (MM) fractions, respectively. Anti-E-CFA antibodies were able to inhibit hemolysis but not cytotoxicity. Hemolytic activity was not affected by heating and a higher reactivity was detected in the carbohydrate-rich fractions, which decreased after reduction by periodate treatment. In infected mice, the fungal load was higher in the early phase, primarily in spleen, liver and brain, with little inflammatory response, with weight loss and presenting behavioral alterations. There was a decreasing in fungal load over the course of infection, although with positive CFU in some organs even after 56 days of infection. There was a gradual increase in the level of anti-*A. kalrae* IgG (IgG1>IgG2a>IgG2b) and high

antigenemia and lower body weight remained during infection. Increased levels of DTH at 14 days were detected, with decrease after this period. There was decreased IFN-γ (14 to 56 days), an increase in IL-4 (7 and 56 days) and increased (7 days) or decreased (56 days) IL-17 levels in the serum. Decreased levels of cytokines were observed in the brain 56 days after infection. We conclude that *A. kalrae* releases soluble factors with cytotoxic and hemolytic activities, with the latter potentially being a thermostable glycoprotein with a high MM, possibly distinct from the cytotoxic factor. We also conclude that *A. kalrae* induces behavioral alteration, weight loss, cellular immunodepression with systemic immunomodulation, possibly to the pattern of Th2 response, inducing cytokine modulation in the brain in experimental infection with *A. kalrae* in mice.

Key-words: hemolysis, cytotoxicity, immune response, cytokines, fungal infection.

LISTA DE FIGURAS

Artigo A:

Artigo B:

LISTA DE SÍMBOLOS, ABREVIATURAS E SIGLAS

AIDS	Acquired Immunodeficiency Syndrome
anti-E-CFA	anti-isogenic erythrocytes sensitized with CFA
BSA	bovine serum albumin
CFA	cell-free antigens
CFU	colony-forming units / unidades formadoras de colônias
CNS	central nervous system
DEAE-celulose	dietilaminoetil-celulose
DTH	delayed-type hypersensitivity / hipersensibilidade tipo tardio
ELISA	Enzyme Linked Immunosorbent Assay
EUA	Estados Unidos da América
FBS	fetal bovine serum
i.p.	intraperitoneal
i.v.	intravenosa / intravenously
HE	hematoxylin-eosin
kDa	kilodalton
MM	massa molecular / molecular mass
MTT	(3-(4,5-di-methylthiazolyl-2)-2, 5-diphenyl-tetrazolium bromide
O.D.	optical density
PBS	phosphate-buffered saline
PDA	potato dextrose agar
PCM	paracoccidioidomycosis
p.i.	post infection
PMSF	phenylmethanesulfonyl fluoride
USA	United States of America

SUMÁRIO

1. INTRODUÇÃO

Arthrographis kalrae é um fungo dimórfico, cosmopolita, neurotrópico, descrito como um raro patógeno em humanos. A patogenicidade do fungo e seu efeito neurológico já foram demonstrados em camundongos infectados, causando síndrome neurológica e lesões principalmente no cérebro e rim. Em humanos, o fungo já foi descrito em diversas manifestações clínicas, como queratites, eumicetoma, onicomicoses, sinusites e meningite, infecção pulmonar, endocardite e encefalite, podendo ser fatal.

No entanto, seus fatores de virulência e mecanismo de patogenicidade ainda não estão esclarecidos. A atividade proteolítica do fungo parece ter um papel importante na patogênese. Foi demonstrado em encefalite micótica em camundongos, que a forma invasiva do fungo é a fase micelial, sendo capaz de penetração vascular através das células endoteliais.

Como este fungo é pouco conhecido e estudado, muitas vezes seu diagnóstico e tratamento são tardios ou errôneos, desencadeando graves consequências para o paciente. Considerando o caráter emergente, é importante conhecer os fatores de virulência assim como a interação parasito-hospedeiro. O seu estudo contribuirá para melhor compreensão do mecanismo de patogenicidade das diferentes manifestações clínicas da doença.

Os objetivos deste trabalho incluem caracterizar melhor os fatores de virulência relacionados às atividades hemolítica e citotóxica de *A. kalrae*, e estudar a resposta imune na infecção experimental murina.

2. OBJETIVOS

2.1. OBJETIVOS GERAIS

Caracterizar parcialmente os fatores hemolíticos e citotóxicos de *Arthrographis kalrae* e estudar a resposta imune celular e humoral na infecção experimental murina.

2.2. OBJETIVOS ESPECÍFICOS

Verificar atividade hemolítica e citotóxica de antígenos solúveis totais de *A. kalrae*.

Caracterizar parcialmente os fatores citotóxicos de *A. kalrae*.

Caracterizar parcialmente os fatores hemolíticos de *A. kalrae*.

Obter anticorpos específicos aos componentes responsáveis pela hemólise.

Avaliar o grau de infecção e o efeito patológico (comportamental e peso) no decorrer de infecção de camundongos com *A. kalrae*.

Avaliar a resposta imune humoral no decorrer de infecção experimental de camundongos com *A. kalrae*.

Avaliar a resposta imune celular no decorrer de infecção experimental de camundongos com *A. kalrae*.

Determinar os níveis de imunocomplexos circulantes no decorrer de infecção experimental de camundongos com *A. kalrae*.

Determinar os níveis séricos de sub-classes de IgG anti-*A. kalrae* e de citocinas (IFN-γ, IL-17, IL-4 e IL-10) no decorrer de infecção experimental de camundongos com *A. kalrae*.

Determinar os níveis de citocinas (IFN-γ, IL-17, IL-4 e IL-10) no cérebro de camundongos infectados com *A. kalrae*.

3. REVISÃO DE LITERATURA

Arthrographis kalrae pertence ao filo Ascomycota, classe Euascomicetos, ordem Eurotiales e família Eremomycetaceae. *Arthrographis* spp. caracteriza-se por hifas conidiogênicas, ramificadas ou não, e conidióforos dendríticos, formando artroconidios, sem células separadas, disjuntores ou conexões entre conídios (Sigler e Carmichael, 1983). A fase anamórfica (assexual) consiste de cinco espécies: *A. cuboidea*, *A. lignicola*, *A. pinicola*, *A. alba* e *A. kalrae*, sendo que as três primeiras ocorrem em madeira, enquanto que *A. alba* foi isolada de sedimentos marinhos e de excrementos de carneiro (Gené *et al*., 1996). A fase teleomórfica de *A. kalrae* corresponde a *Eremomyces langeronii* (Malloch e Sigler, 1988).

A. kalrae foi descrito anteriormente por Cochet (1939) e por Tewari e Macpherson (1971) como *Arthrographis langeroni* e *Oidiodendron kalrai*, respectivamente. Posteriormente, Sigler e Carmichael (1983) juntaram as duas espécies, reclassificando como *Arthrographis kalrae* com base nos conidióforos e conídios (Sigler e Carmichael, 1983; Tewari e Macpherson, 1971).

A. kalrae é um fungo dimórfico, sendo temperatura e nutrição-dependente, com a fase micelial predominante no tecido infectado (Tewari e Macpherson, 1968; 1971). O fungo se apresenta na fase filamentosa em temperatura ambiente (25 ºC) e na fase leveduriforme a 37 ºC. A fase filamentosa consiste de hifas septadas, ramificadas ou não, com artroconidios ovais a redondos em cadeia na extremidade de conidióforos arborescentes ou não ramificados, com ocasionais clamidoconidios uni ou multicelulares. As colônias brancas ou cor creme apresentam-se com aspecto aveludado ou com textura poeirenta a granular. A fase leveduriforme apresenta-se como células ovais ou elípticas, podendo ocorrer uma mistura de blastoconidios, artroconidios e até hifas septadas. Macroscopicamente, observaram-se colônias de cor creme, com aparência enrugada, passando a aveludadas após três a sete dias de incubação. O fungo também cresce a 42 ºC (Tewari e Macpherson, 1968; 1971; Chin-Hong *et al*., 2001; Xi *et al*., 2004; Pichon *et al*., 2008).

Dentre as características bioquímicas, salienta-se a hidrólise de uréia em 24 h e de caseína em 48 h a 37 ºC, sendo a hidrólise da caseína maior na fase micelial. *A. kalrae* não fermenta glucose, galactose, maltose, lactose, sacarose, salicina, adonitol, rafinose, trealose e inulina (Tewari e Macpherson, 1971).

A. kalrae vem sendo descrito como um raro patógeno em humanos. Originalmente isolado de lesões em humanos e animais (como cachorro e cavalos), também foi isolado de escarro e aspirado de lesão pulmonar de uma mulher na Nova Zelândia na década de 80 (Sigler e Carmichael, 1983).

Na França, um caso de eumicetoma na mão causado por *A. kalrae* ocorreu e foi curado com itraconazole após quatro meses de terapia (Degavre *et al*., 1997).

Em um estudo avaliando a incidência de sinusite fúngica alérgica em pacientes com rinosinusite crônica, *A. kalrae* foi identificado em 0,5% dos pacientes (Ponikau *et al*., 1999).

Chin-Hong *et al*. (2001) descreveram um caso de pansinusite e meningite com *A. kalrae* envolvendo um paciente com AIDS, nos EUA. Ele estava sendo tratado com itraconazole, porém foi a óbito devido à pneumonia por *Pneumocystis carinii*.

Dois casos de queratites foram descritos nos EUA em pacientes apresentando intensa fotofobia. As manifestações clínicas e os primeiros achados clínicos davam forte impressão de infecção por *Acanthamoeba*, mas a infecção por *A. kalrae* foi confirmada. Em ambos os casos os pacientes relataram envolvimento com atividades de jardinagem e envolviam uso de lente de contato, sugerindo que esta pode ter facilitado a invasão do fungo (Perlman e Binns, 1997; Biser *et al*., 2004).

Na China, foi descrito um caso de doença causada por *A. kalrae*, em 2004, em paciente aparentemente saudável anteriormente a trauma em olho esquerdo. Ele apresentou panoftalmite e sinusite invasiva envolvendo os seios maxilar e etmóide. Após tratamento com agentes antibacterianos e esteróides, os sintomas pioraram, quando então o agente causal foi identificado e o paciente tratado com antifúngicos. No entanto, foi necessária uma intervenção cirúrgica e o paciente perdeu a visão no olho acometido (Xi *et al*., 2004).

Em 2008, foram descritos dois casos envolvendo o fungo. Na Eslováquia, esse micro-organismo foi isolado de dois pacientes que

apresentavam onicomicose (Volleková *et al*., 2008). E na França, Pichon *et al*. (2008) descreveram um caso de sinusite aguda e diagnóstico de pneumonia por *Streptococcus pneumoniae* mas que posteriormente apresentou sinusite, hemiplegia e acidente vascular cerebral, levando à morte encefálica. Exames revelaram infecção fúngica no sistema nervoso central, com necrose e trombose, responsáveis pelo infarto e acidente vascular cerebral, com isolamento de *A. kalrae* do fluido cérebro-espinhal.

Sugiura e Hironaga (2010) descreveram um caso de onicomicose no Japão. Os autores isolaram o fungo do solo da casa do paciente e em mais de 60% dos solos comerciais, sugerindo que o solo seja a fonte de infecção. No mesmo ano, De Diego Candela *et al*. (2010) descreveram um caso de endocardite causado por *A. kalrae* na Espanha, que necessitou de vários procedimentos cardíacos, resultando em morte do paciente. Na Alemanha, em 2011, houve um caso grave de infecção de córnea, em paciente em uso de lentes de contato, que necessitou de várias ceratoplastias, mesmo após longo tratamento com antifúngico (Thomas *et al*., 2011).

Os casos recentes envolvendo *A. kalrae* foram descritos em 2012, envolvendo uma grave infecção pulmonar na Holanda, resolvida com cirurgia e tratamento antifúngico (Vos *et al*., 2012), e uma infecção no joelho na Austrália (Boan *et al*., 2012).

Tewari e Macpherson (1968) demonstraram a patogenicidade de *A. kalrae* e seus efeitos neurológicos em camundongos infectados com a fase leveduriforme, observando-se uma síndrome neurológica complexa, com hiperirritabilidade, ataxia, giros e saltos. A taxa de mortalidade atingiu 60% quando a inoculação foi via intravenosa (i.v.) e 10% por via intraperitoneal (i.p.). O efeito produzido pela via i.v. apresentou-se mais grave em relação à via i.p., embora a quantidade de células inoculadas no primeiro grupo ($3{,}5 \times 10^7$ células) fosse quase a metade da quantidade inoculada no último grupo. Lesões foram demonstradas nos rins, cérebro e baço, com focos necróticos e infiltrado inflamatório. Elementos fúngicos (artroconidios e blastoconidios e/ou células leveduriformes) foram encontrados nesses órgãos além do fígado e pulmões.

Swenberg *et al*. (1969a) usaram *A. kalrae* como modelo experimental de encefalite micótica e demonstraram a síndrome neurológica e lesões no

sistema nervoso central de camundongos infectados com fase leveduriforme, confirmando os resultados anteriores. O micélio penetrou rapidamente nos vasos sangüíneos e tecidos adjacentes, indicando que esta fase seja sua forma invasiva; e os artroconidios representaram a forma mais resistente à resposta inflamatória do hospedeiro. No modelo também observou-se diferença entre camundongos tratados ou não com cortisona, uma vez que a cortisona deprime o sistema imune, simulando as circunstâncias em que as infecções micóticas ocorrem em seres humanos. A proliferação e invasão do fungo no grupo não tratado diminuíram com o aumento da resposta celular; já o grupo tratado com cortisona apresentou maiores taxa de mortalidade e proliferação do fungo, continuando as estruturas fúngicas intactas.

A patogenicidade de *A. kalrae* na encefalite micótica experimental foi investigada por microscopia eletrônica. Em camundongos normais, a proliferação do fungo foi maior no segundo dia após infecção e, no terceiro, com edema, áreas de vasculite, e células necróticas, mas com nódulos de células mononucleares e polimorfonucleares, raramente o fungo estava presente, uma vez que o sistema imune conseguiu controlar a proliferação fúngica. As lesões se tornaram menos frequentes no quarto e quinto dias, e nenhuma lesão foi observada a partir do sétimo dia. Já em camundongos tratados com cortisona, a proliferação fúngica dentro de capilares foi demonstrada já com 8 h após infecção. Os cérebros apresentavam lesões hemorrágicas, necrose e edema, entre o quarto e sexto dia. Uma vez dentro do cérebro, o fungo parecia capaz de crescer em todas as direções. A penetração vascular se deu pela extensão da hifa entre as células endoteliais. Fagocitose e digestão do fungo por astrócitos e células mono e polimorfonucleares foram demonstradas (Swenberg *et al*., 1969b).

Embora o mecanismo de infecção de *A. kalrae* ainda não esteja totalmente elucidado, a habilidade de formar micélio parece ser um importante fator na patogênese, uma vez que essa fase representa sua forma invasiva em vasos e tecidos, sendo que os artroconidios representam a forma mais resistente à resposta inflamatória do hospedeiro, além de a penetração vascular ocorrer pela extensão da hifa entre as células endoteliais (Swenberg *et al*.,1969a e 1969b).

O perfil proteolítico intracelular constou da hidrólise de caseína,

hemoglobina, gelatina, lactalbumina, orceína-elastina, colágeno e membrana basal de rim de coelho. A purificação dessas proteases combinando métodos de precipitação em sulfato de amônio, cromatografia em Sephadex G-200 e DEAE-celulose, permitiu identificar um complexo de pelo menos seis enzimas proteolíticas intracelulares. A atividade proteolítica provavelmente desempenha um papel importante na patogênese de *A. kalrae*, ainda mais considerando que o micro-organismo é encontrado associado aos tecidos do hospedeiro, indicando participação dessas proteases no processo infeccioso (Cino e Tewari, 1975; 1976).

Nos últimos anos o número de casos de *A. kalrae* relatados vem crescendo. No entanto, ainda existem poucos dados na literatura, tornando importante a investigação, tanto pelo fato desse fungo atuar como patógeno em pacientes imunocompetentes e também como oportunista, podendo ser fatal. Além disso, inexiste na literatura estudo relacionado à resposta imune a *A. kalrae*. O estudo dos fatores de virulência e da resposta imune no decorrer da infecção contribuiria no entendimento da interação fungo-hospedeiro, avançando no diagnóstico preciso e rápido.

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5. ARTIGO A: *Arthrographis kalrae* soluble antigens present hemolytic and cytotoxic activities

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Abstract

Arthrographis kalrae is a dimorphic, cosmopolitan and neurotropic fungus that has been described as a rare human pathogen. This study investigated the hemolytic and cytotoxic activities in *A. kalrae* cell-free antigens (CFA). Total CFA and its Sephadex chromatography fractions were tested on mouse erythrocytes for hemolysis and on a P3U1 cell line for cytotoxicity. Hemolytic and cytotoxic activities were detected in distinct molecular mass (MM) fractions. Additionally, antibodies against isogenic erythrocytes sensitized with CFA (anti-E-CFA) were able to inhibit hemolysis but not cytotoxicity. Hemolysis was not affected by heating and a higher reactivity was detected in the carbohydrate-rich fractions, which decreased after reduction by periodate treatment. The pioneering nature of this work is due to the demonstration of the cytotoxic activity in *A. kalrae* and the suggestion that this activity may be due to molecules distinct from the hemolytic factor, with the latter potentially being a thermostable glycoprotein with a high MM.

Keywords: hemolysis, cytotoxicity, fungus, chromatography, antibodies.

Introduction

The microorganism *Arthrographis kalrae*, a dimorphic fungus that can be isolated from soil, has been described as a rare human pathogen. As this fungus is barely known and rarely studied, its diagnosis and treatment are frequently delayed or erroneous. *Arthrographis kalrae* has been described in the following cases: eumycetoma of the hand in France [1], sinusitis and meningitis in an AIDS patient in the USA [2] and panophthalmitis and invasive sinusitis involving the maxillary and ethmoid sinuses in the People's Republic of China [3]. In addition, cases of keratitis that initially strongly suggested *Acanthamoeba* have been described in the USA [4, 5]. There have been cases of onychomycosis in Slovakia [6] and Japan [7], cerebral vasculitis leading to fatal-stroke syndrome in France [8], endocarditis in Spain [9] and an eye infection in an immunocompetent patient in Germany [10]. The most recent cases reported in the literature involved a pulmonary infection in the Netherlands [11] and a knee joint infection in Australia [12], both occuring in 2012.

Its pathogenicity and neurological effects were demonstrated in infected mice, with this pathogen causing a complex neurologic syndrome and lesions mainly in the brain and kidneys [13]. The mechanism of infection by *A. kalrae* is unknown. The mycelial phase has been demonstrated to represent the invasive form of the fungus in experimental mycotic encephalitis, with the arthrospores representing the form most resistant to the host's inflammatory response [14]. The proteolytic activity of the fungus, involving a complex of at least 6 proteolytic enzymes, may play a relevant role in its pathogenicity [15, 16].

Few data exist in the literature about the virulence factors of *A. kalrae*, which requires further investigation because this fungus acts as an important pathogen even in immunocompetent patients, may be associated with several other diseases related to immunosuppression and can be fatal. Such research could contribute to the study of host-parasite interactions and lead to improved diagnosis. This study thus aimed to investigate this pathogen's hemolytic and cytotoxic activities, which may be essential virulence factors of the fungus.

Materials and Methods

Microorganism and cell-free antigen (CFA) preparation

The fungus *A. kalrae* (IFM55165) was isolated from a skin lesion of a cat and was provided by the Research Center of Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan. CFAs were obtained according to Camargo et al. [17], with some modifications. *Arthrographis kalrae* was grown on 4% Sabouraud glucose agar (Acumedia, Lansing, MI, USA) at 35 °C for 5 days. The fungus was then collected and suspended in 0.15 M phosphate-buffered saline (PBS) at pH 7.2, with 0.02% thimerosal and 2.5 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA). After mixing with a vortex-mixer for 5 min, the samples were allowed to stand for 1.5 h at 4 °C. Then, the samples were mixed again for 5 min, homogenized and centrifuged at 1005 x *g* at 4 °C for 15 min (Eppendorf Centrifuge, Hamburg, Germany). The supernatant was centrifuged at 12870 x *g* at 4 °C for 20 min. The supernatant (CFA) was collected, and the protein concentration, which was determined by the Folin method, was adjusted to 3 mg of protein/mL.

Hemolysis test with native and heated CFA forms

Mouse blood was collected in Alsever's solution and the erythrocytes were washed 3 times with PBS (centrifugation at 175 x *g* at 4 °C for 10 min; Eppendorf Centrifuge, Hamburg, Germany). A 1% (vol/vol) erythrocyte suspension was prepared with PBS. CFA and heated CFA (56 °C, 30 min) samples were diluted in series (ratio 1:2) and then incubated 1:1 with a 1% erythrocyte suspension in "V" bottom plates for 3 h at 35 °C. PBS was used as a negative control, and distilled water as a positive control for hemolysis. The plates were centrifuged at 155 x *g* at 4 °C for 10 min, and the supernatants were collected and analyzed by absorbance reading at 550 nm (Labsystems, Helsinki, Finland).

Cytotoxicity assay with CFA

Mouse myeloma cells (cell line P3U1) were seeded on 96-well culture plates at 0.5 x 10^6 cells/mL in RPMI medium supplemented with 10% fetal bovine serum (FBS). CFAs were added at concentrations varying from 0 to 1000 μg/mL, and the cells were incubated at 37 °C for 30 min and 1, 2, 4, 8, 12, 24 and 48 h. The cytotoxic effect was evaluated with the (3-(4,5-di-methylthiazolyl-2)-2, 5-diphenyl-tetrazolium bromide (MTT) method according to the manufacture'sr instruction (TACS® MTT Cell Proliferation Assay, Trevigen, Inc, Helgerman Ct. Gaithersburg, MD, USA).

Sephadex G-150/120 gel filtration chromatography

CFA samples (3-4 mL) were applied to a Sephadex G-150/120 column (0.65 x 30 cm) (Sigma, St. Louis, MO, USA) and balanced with PBS using PBS as the eluent. Two-milliliter fractions were collected with an automatic fraction collector (Gilson Inc., Middleton, WI, USA), and the absorbances were read at 280 nm. For these tests, the CFA fractions were dialyzed against distilled water for 48 h at 4 °C, lyophilized and suspended in PBS at a concentration of 3 mg/mL.

Hemolysis test with chromatography fractions

This test was performed under the same conditions described above. The fractions were incubated 1:1 with a 1% mouse erythrocyte suspension for 3 h at 35 °C. The plates were centrifuged at 155 x *g* at 4 °C for 10 min, and the supernatants were collected and analyzed at 550 nm (Labsystems, Helsinki, Finland). PBS was used as the negative control, and CFA as the positive control. The absorbance readings of the fractions diluted in PBS (1:1) at 550 nm were subtracted from the sample readings after the hemolysis test, and the results were subjected to statistical analyses. The hemolysis test was also performed on a 3% agar blood plate. Wells were made and filled with the CFA fractions (40 μL), and the plate was incubated for 3 h at 35 °C.

Cytotoxicity test with chromatography fractions

The Sephadex G-150/120 chromatography fractions were tested individually from 25 to 250 μg/mL for 2 h with the same method described above.

Immunization of mice with isogenic erythrocytes sensitized with CFA (E-CFA)

This study was approved by the Animal Experimentation Ethics Committee of the State University of Londrina. Balb/c mouse blood was initially collected in Alsever's solution, and the erythrocytes were washed 3 times with PBS. A 5% erythrocyte suspension was prepared with PBS. A CFA sample in a sublytic dilution (1/2), was incubated 1:1 with the 5% erythrocyte suspension for 1 h at 35 °C and 1 h at 4 °C. The erythrocytes were washed 3 times with Hank's solution and resuspended in the same solution. An emulsion with Freund's adjuvant (complete or incomplete) was then prepared for immunization of the Balb/c mice. The first dose was performed with complete Freund's adjuvant (Sigma, St. Louis, MO, USA) and the second and third doses with incomplete Freund's adjuvant (Sigma, St. Louis, MO, USA) at successive two-week intervals. After the third dose, double radial immunodiffusion analysis was done.

Hemolysis inhibition test with anti-E-CFA antibodies

Anti-E-CFA and normal sera were initially inactivated at 56 °C for 30 min. CFA, anti-E-CFA serum (1:40) and erythrocytes (1%) were incubated at 35 °C for 3 h in a "V" bottom plate. The plate was centrifuged at 155 x *g* for 10 min at 4 °C (Eppendorf Centrifuge, Hamburg, Germany). The supernatants were collected and analyzed at 550 nm (Labsystems, Helsinki, Finland). Normal serum was used as a control. Pure CFA and PBS were used as positive and negative controls for hemolysis, respectively.

Cytotoxicity neutralization test with anti-E-CFA antibodies

Anti-E-CFA or normal serum (1:40), CFA (250 μg/mL) and P3U1 cells (0.5 x 10^6 cells/mL) were incubated at 37 °C for 2 h. CFA only and cells were used as a

positive control; PBS and cells were used as a negative control. Cytotoxicity was evaluated by the MTT method, as described above.

Determination of carbohydrate level in Sephadex chromatography fractions

The carbohydrate level of the Sephadex fractions was analyzed by the phenol-sulfuric acid method in a microplate. The analyses were performed with 7.5 μg of protein in each fraction. The total carbohydrate level was determined by spectrophotometry at 492 nm using a D-mannose standard curve.

Comparative analysis of the CFA fraction reactivity with anti-E-CFA antibodies by ELISA and ELISA using periodate

Plates were coated with Sephadex chromatography fractions (5 μg/mL) in a carbonate buffer, pH 9.6, for 1 h at 35 °C and overnight at 4 °C. The plates were washed 4 times with wash buffer (PBS containing 0.05% Tween 20 and 0.5% skim milk), blocked with blocking buffer (PBS containing 0.05% Tween 20 and 5% skim milk) and washed 3 times with 50 mM sodium acetate, pH 4.5. Sodium periodate in a sodium acetate buffer or just acetate buffer alone was then added to each well, and the plates were incubated for 1 h at room temperature. After washing 3 times, the plates were incubated with 0.1 M Tris at pH 7.2 in 0.5% glycerol for 30 min at 35 °C, followed by incubation with 1% glycine for 1 h at 35 °C. The plates were washed 3 times with PBS, incubated with anti-E-CFA antibodies diluted 1:200 for 2 h at 35 °C, washed 4 times with wash buffer and incubated with goat anti-mouse IgG labeled with peroxidase (Sigma, St. Louis, MO, USA) diluted 1:4000 for 1.5 h at 35 °C. After being washed, the reaction was revealed with a substrate solution (5 mg ortho-phenylenediamine, 10 mL 0.1 M citrate buffer, pH 4.5 and 10 μL H_2O_2) and blocked with 4 N H_2SO_4.

Statistical Analysis

The data were analyzed by GraphPad Prism 6.0 software (GraphPad Software, Inc, La Jolla, CA, USA) by using ANOVA and Tukey's test or t-test; we considered $p<0.05$ to be statistically significant.

Results

Hemolysis test with antigens: CFA and heated CFA

The results showed hemolysis of mouse erythrocytes by both pure and diluted 1:2 (final concentrations of 1500 and 750 μg/mL, respectively) CFA. Hemolytic activity was maintained even after heating the sample for 30 min at 56 °C, indicating thermostability (Fig. 1).

Cytotoxicity test with CFA

The results with CFA at various concentrations and times showed that at lower antigen concentrations (10 to 25 μg/mL), the percentage of dead cells was less than 20% and showed a progressive increase at intermediate concentrations (50 to 100 μg/mL). Above 250 μg/mL, the percentage of dead cells was greater than 70% regardless of the incubation time (Fig. 2).

Hemolysis and cytotoxic test with Sephadex G-150/120 chromatography fractions

The quantitative analysis of hemolysis using the chromatography fractions demonstrated lysis with fractions 8 – 13, 15 – 17 and 27 – 30. We found 3 peaks of cytotoxicity. The highest peak was associated with the fractions corresponding to the second peak of the chromatography column, which reached more than 70% mortality at the highest concentration tested. The spectrophotometric profile of the CFA Sephadex G-150/120 fractions and the quantitative analyses of hemolysis and cytotoxicity are shown in Fig. 3. A hemolysis test on a blood agar plate showed lysis with fractions from 7 – 16 and 29 and 30 (data not shown).

Hemolysis and cytotoxicity inhibition test with anti-E-CFA antibodies

We found a decrease in hemolysis of 86% with anti-E-CFA compared with normal serum. Hemolysis was 9.2% when anti-E-CFA antibodies were added compared with 70% with normal serum. We observed no difference in

cytotoxicity when anti-E-CFA or normal serum was added to the cells with CFA (Fig. 4).

Determination of total carbohydrates

The total carbohydrate amount in each fraction was analyzed and showed that the higher molecular mass fractions, which correspond to the first peak of chromatography, have increased carbohydrate levels (Fig. 5).

Comparative analysis of CFA fraction reactivity with anti-E-CFA antibodies by ELISA and by ELISA using periodate

Antigens treated with periodate exhibited a reduction in the reactivity of anti-E-CFA antibodies. This reduction was higher than 70% from fraction 7 to 15 (Fig. 5).

Discussion

Hemolytic activity was demonstrated in mouse erythrocytes, with lysis observed only in concentrated samples, suggesting a low activity or low concentration of factors responsible for the lysis. This activity may play an important role in the pathogenicity of the fungus and may possibly act regardless of the species. The first evidence of hemolytic activity in *A. kalrae* was reported in 2007 [18] for sheep erythrocytes. Hemolysis may be a relevant virulence factor as iron is an essential nutrient for most organisms and the concentration of free iron in mammalian hosts is extremely low for the growth of microorganisms. One of the mechanisms developed for the acquisition of iron from the host is the use of the heme component released after the lysis of erythrocytes [19]. Studies with *Candida albicans* indicated that this microorganism must acquire iron from the production of a factor present in the fungus and secreted into the culture medium that releases hemoglobin by the lysis of erythrocytes [20].

Studying hemolytic activity is fundamental because, in addition to their role in oxygen transport in the organism, erythrocytes also play an important

role in binding to circulating immunocomplexes and transporting them to the liver and spleen [21]; the decrease in erythrocyte levels could interfere with several systems. No studies have been performed on the presence of immunocomplexes in *A. kalrae* infections. However, given that CFA contains soluble antigens that present immunogenicity/antigenicity [18], in cases of infection by *A. kalrae*, the formation of immunocomplexes could occur, thereby contributing to its pathogenicity.

Hemolytic activity was not inactivated even after heating at 56 ºC for 30 min, which was expected as the fungus is able to grow at high temperatures (42 °C) [2, 3, 8]. In addition to the observation that the component was not inactivated by heating, hemolytic activity was not inhibited by the addition of PMSF, a protease inhibitor, indicating that the hemolytic component was most likely not a protease.

To verify whether *A. kalrae* had cytotoxic effects, as a first step, we tested total antigens at various concentrations and incubation times and detected an effect at 10 μg/mL, which reached its maximum activity at 250 μg/mL of CFA in 30 min. In the second step, during chromatography fraction analysis, we detected activity in the second peak, corresponding to the low MM fractions.

Such cytotoxic components may contribute to the pathogenicity of *A. kalrae* and perhaps represent relevant virulence factors requiring further studies. Many microorganisms have cytotoxic effects, whether they are bacteria such as *Campylobacter pylori* or fungi such as *Aspergillus* and *Penicillium* [22, 23].

Hemolysins are proteins that lyse erythrocytes and nucleated cells. Although more extensively investigated in bacteria, they also have been studied in some fungi [24]. In our study, the cytotoxic components may be not the same as those having hemolytic activity because the fractions with these activities are distinct. Cytotoxicity appears to be caused by components with a low MM and hemolysis by high MM antigens. Moreover, many of the hemolysins in fungi have a low MM [24, 25]. However, in our study, the factor(s) responsible for hemolysis appear to have a high MM. This finding is more in accord with the work of Watanabe et al. (1999), which characterized a hemolytic factor of approximately 200 kDa from *Candida albicans* [26].

For a better characterization of the hemolytic components, Balb/c mice were immunized with isogenic erythrocytes sensitized with CFA. Through this process, the components responsible for hemolysis can be selected, making possible the production of specific antibodies against these hemolytic factors. The hemagglutination activity of *Histoplasma capsulatum* was studied using erythrocytes sensitized with soluble antigens to obtain polyclonal antibodies against the hemagglutinating antigen. These antibodies inhibited hemagglutination, thereby being specific and demonstrating that this technique is useful in characterizing these components [27]. Our anti-E-CFA antibodies inhibited hemolysis, resulting in a significant decrease compared with normal serum and suggesting that anti-E-CFA has specific antibodies against the hemolytic components of *A. kalrae*. Inhibition of hemagglutination and hemolysis by monoclonal antibodies was also demonstrated in *Porphyromonas gingivalis* [28].

These antibodies were also tested to determine whether they could inhibit cytotoxicity. Anti-E-CFA antibodies did not inhibit cell death by CFA. When the components causing hemolysis are different from those causing cytotoxicity, anti-E-CFA antibodies cannot interfere with the cytotoxic effects.

The polyclonal antibodies obtained in this work allowed us to recognize fractions corresponding to the first peak and intermediary fractions of Sephadex chromatography by ELISA, which were compatible with the fractions presenting hemolytic activity. The non-detection of fractions corresponding to the second peak of Sephadex column by ELISA may be due to their low MM and consequently low immunogenicity. However, the last fractions presented hemolytic activity and were detected by ELISA. It was due to the high MM components present in these fractions that were demonstrated by electrophoresis (data not shown), possibly because of contamination.

As discussed above, the hemolytic factor(s) did not appear to be enzymatic. Therefore, to determine whether the biological activity sites were carbohydrates, we treated them with periodate. ELISA using periodate-treated antigens showed that the antibodies, mainly anti-E-CFA, recognize carbohydrate antigens. Furthermore, the carbohydrate level was determined by the phenol-sulfuric acid method, showing that the fractions with hemolytic activity have a high carbohydrate content. Thus, we suggest that the hemolytic

components are glycoproteins. This is also in agreement with the work of Watanabe et al. (1999), which reported that the hemolytic factor from *C. albicans* may be a mannoprotein [26].

In conclusion, we report that *A. kalrae* produces both hemolytic and cytotoxic factors. We suggest that the hemolytic factor is most likely a glycoprotein and possibly one with high MM. However, the cytotoxic components appear to have a low MM, suggesting that the hemolytic and cytotoxic factors are distinct. The more specific antibodies obtained in this work will contribute to future studies on host-fungus interactions and will also improve diagnostic capabilities. As an emerging fungal pathogen, a better characterization of this agent is increasingly important.

Conflict of interest statement:

The authors have no financial conflict of interest.

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Figures

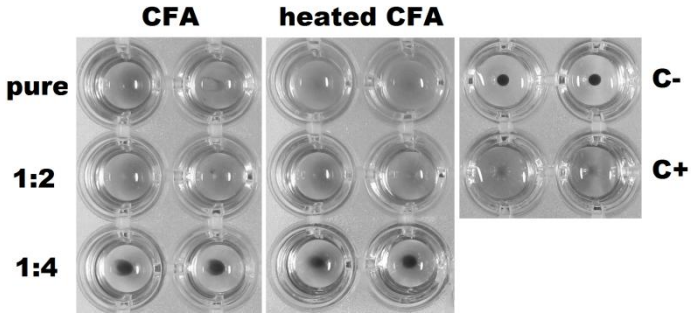

Figure 1. Hemolysis test with native and heated CFA. The native and heated CFA (56 °C, 30 minutes), pure and diluted 1:2 and 1:4, were incubated in duplicate with a 1% mouse erythrocytes. C-: negative control (PBS); C+: positive control (distilled water).

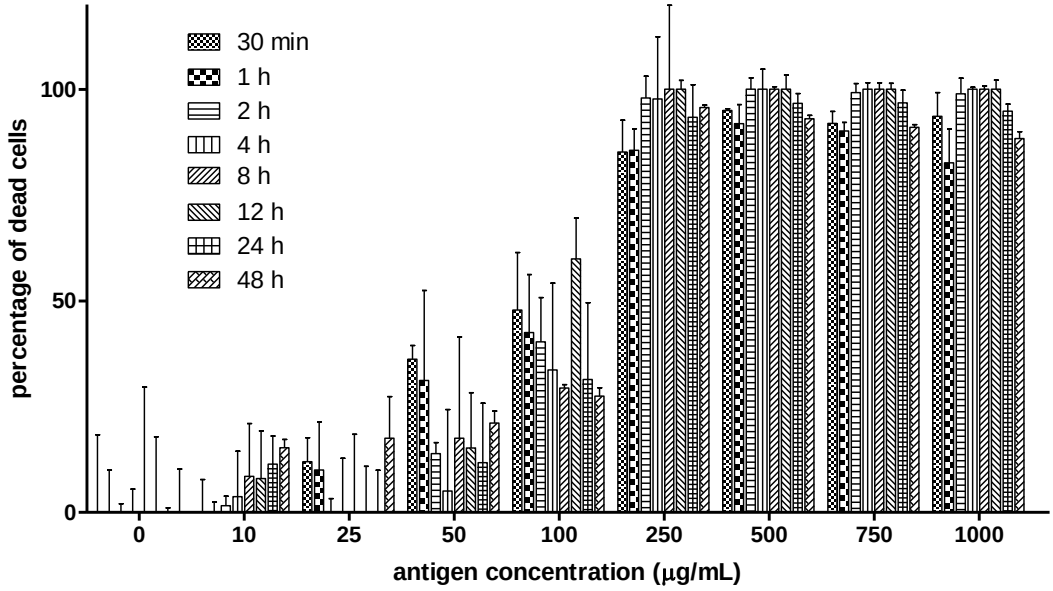

Figure 2. Cytotoxicity test with CFA. The percentage of dead cells is shown at different concentrations of *A. kalrae* CFA and incubation times.

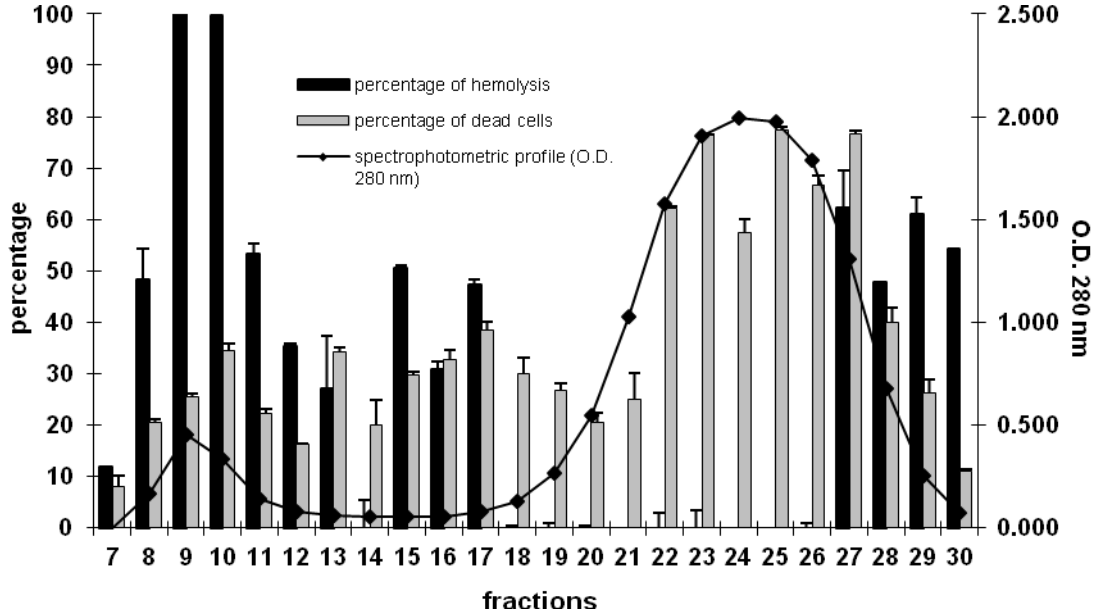

Figure 3. Spectrophotometric profile of *A. kalrae* CFA chromatography, hemolysis and cytotoxicity test with the fractions. CFA chromatography was performed with Sephadex G-150/120 and fractions were read at 280 nm. Quantitative analysis of the hemolysis of the fractions in percentage demonstrated hemolytic activity in fractions 8 – 13, 15 – 17 and 27 – 30 ($p<0.05$); and cytotoxicity test with fractions at 250 μg/mL for 2 h showed 3 peaks of cytotoxicity, in percentage of dead cells, with the highest one corresponding to the second peak of the spectrophotometric profile of chromatography.

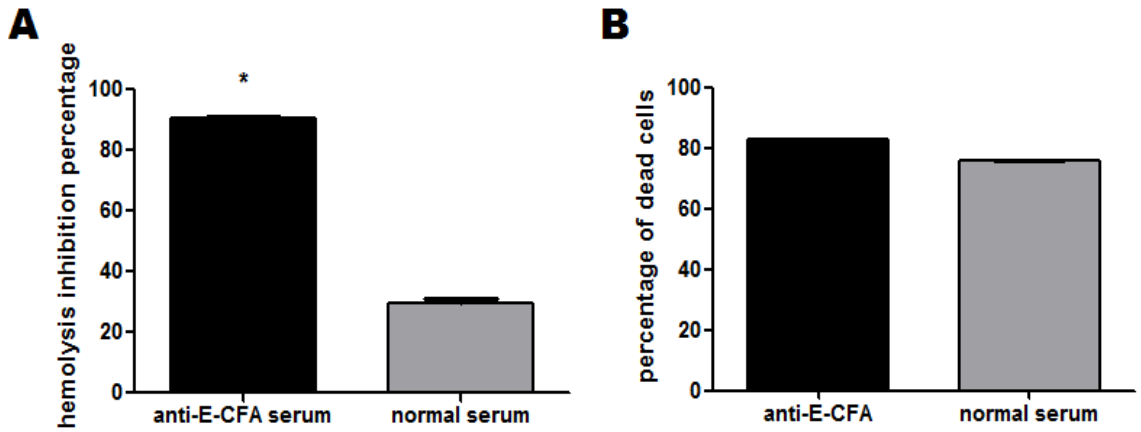

Figure 4. Hemolysis and cytotoxicity inhibition tests. Hemolysis inhibition (A) and cytotoxicity neutralization (B) tests were performed using anti-E-CFA and normal serum. The antigen (CFA) without antibodies was considered to be 100% hemolysis. * $p<0.05$.

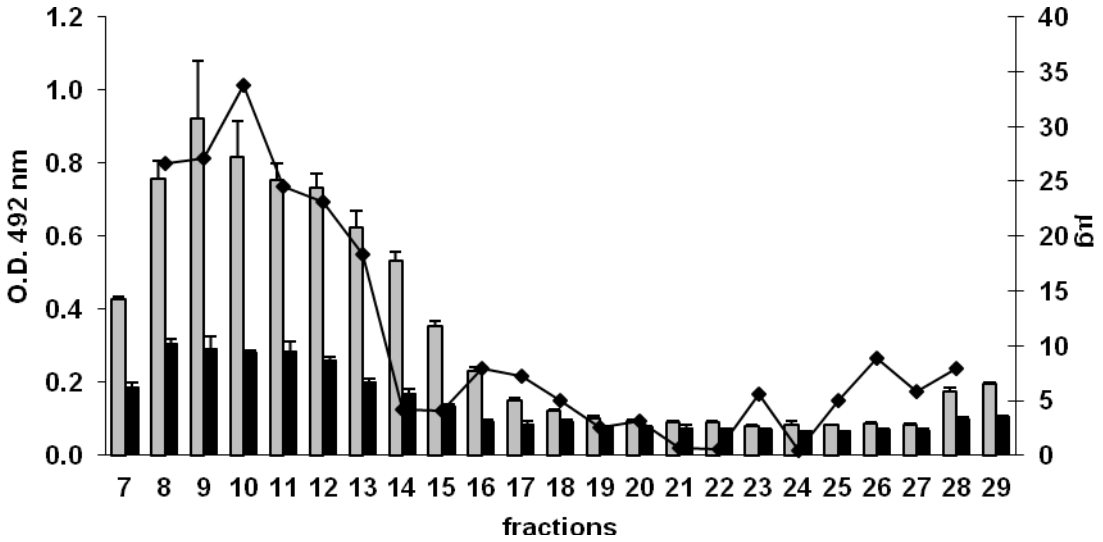

Figure 5. Carbohydrate levels in the *A. kalrae* CFA fractions and reactivity of the fractions treated or not with periodate with anti-E-CFA antibodies. Anti-E-CFA antibodies were tested by ELISA using Sephadex G150/120 CFA fractions treated (black bars) or not (gray bars) with sodium periodate, showing a reduction in the reactivity of the treated fractions ($p<0.05$). The line shows the carbohydrate levels in the fractions from Sephadex chromatography. The amount of carbohydrates was evaluated in 7.5 μg of protein in each fraction.

6. ARTIGO B: Immunomodulation over the course of experimental *Arthrographis kalrae* infection in mice

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Abstract

Arthrographis kalrae is a neurotropic fungus that is occasionally described as an opportunistic human pathogen. This study investigated the immune response to *A. kalrae* during murine experimental infection. Balb/c mice were challenged intravenously with *A. kalrae* ($5x10^6$ cells), and the fungal load (CFU or antigen level) was measured. The humoral (IgG) and cellular (DTH) immune responses induced in response to *A. kalrae* soluble antigens and levels of systemic and cerebral cytokines (IFN-γ, IL-4, IL-10 and IL-17) were evaluated at 7, 14, 28 and 56 days post infection. Infected mice presented with neurological syndrome and weight loss over the course of the infection. The fungal load was higher in the early phase, but gradually decreasing numbers of viable fungi were detected at 56 days. There was a gradual increase in the level of anti-*A. kalrae* IgG (IgG1>IgG2a>IgG2b) and increased levels of DTH at 14 days, which was followed by a reduction in these levels. There was decreased IFN-γ (14 to 56 days), an increase in IL-4 (7 and 56 days) and increased (7 days) or decreased (56 days) IL-17 levels in the serum. Decreased levels of cytokines (IFN-γ, IL-4, IL-10 and IL-17) were observed in the brain at 56 days post infection. The results suggest that the immune response during murine *A. kalrae* infection modulates to the pattern of Th2 response, which could explain the persistence of viable fungus even after a long period of infection. This study

shows for the first time the cytokines and cellular immunomodulation that occur in response to an experimental infection with *A. kalrae* in mice.

Key-words: cytokines, fungal infection, central nervous system, immune response, immunosuppresion.

Introduction

Arthrographis kalrae is a neurotropic, dimorphic fungus that has been described as a rare human pathogen, with diverse clinical manifestations throughout the world. *A. kalrae* was described in a case of eumycetoma in France [1], sinusitis and meningitis and two cases of keratitis in the USA [2-4], panophthalmitis and sinusitis in the People's Republic of China [5], cases of onychomycosis in Slovakia and in Japan [6, 7], endocarditis in Spain [8], eye infection in Germany [9], pulmonary infection in The Netherlands [10] and a knee joint infection in Australia [11]. Pichon et al. (2008) described a case of invasive fungal cerebral vasculitis that resulted in stroke and death in a non-immunocompromised patient [12].

Although the cases are rare, for many of them, the diagnosis and treatment were protracted or erroneous, leading to serious complications in the patient, including death; therefore it is important to investigate the interaction of this fungus with the host.

The pathogenicity of the fungus and its neurological effects have been demonstrated in infected mice, and signs include neurological syndrome and lesions, mainly in the brain and kidneys [13]. Although Swenberg et al. (1969) introduced an experimental model for mycotic encephalitis induced by this fungus [14, 15], there is no data in the literature on the cytokines, local or systemic immune responses that develop during infection by *A. kalrae*.

This study investigated for the first time the fungal load, humoral and cellular immune responses to *A. kalrae* antigens, immune complexes and systemic and cerebral cytokine (IFN-γ, IL-4, IL-10 and IL-17) levels over the course of infection in mice.

Materials and methods

Microorganism

Arthrographis kalrae (IFM55165), isolated from a cat skin lesion, was kindly provided by the Research Center of Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan.

A. kalrae antigens (cell-free antigens – CFA)

CFA were generated according to Camargo et al. [16], with some modifications. *A. kalrae*, grown on 4% Sabouraud glucose agar (Acumedia, Lansing, MI, USA) at 35 °C for 5 days, was collected in 0.15 M phosphate-buffered saline (PBS), pH 7.2, with 0.02% thimerosal and 2.5 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA). After mixing on a vortex-mixer for 5 min, it stood for 1.5 h at 4 °C. It was then mixed again for 5 min and homogenized and centrifuged at 1005 x *g* and 4 °C for 15 min (Eppendorf Centrifuge, Hamburg, Germany). The supernatant was centrifuged at 12870 x *g* and 4 °C for 20 min. The supernatant (CFA) was collected, and the protein concentration was determined by the Folin method.

Experimental protocol

Female Balb/c mice (6 to 8 weeks old) were randomly divided into 4 groups with 11 animals each. Seven mice from each group were intravenously (i.v.) infected with 5 x 10^6 cells in 100 μL PBS. The other animals were inoculated with PBS i.v. in a volume of 100 μL. The groups were sacrificed at 7, 14, 28 or 56 days after infection. The infected mice were monitored daily for behavioral alterations. All of the procedures applied to the animals in this study were approved by the Animal Care and Use Committee of the State University of Londrina (CEEA nº 82/09).

CFU

For CFU determination, organs (spleen, liver, kidney, lung, heart and brain) were aseptically removed, weighted and homogenized in PBS (0.2 g of tissue/mL). The homogenates (50 μL) were plated on potato dextrose agar (PDA) plates (Himedia, Mumbai, India), and the colonies were counted after 48 h of incubation at 35 ºC. The results are expressed as $\log_{10}$ CFU/g of each organ. After the addition of a protease inhibitor cocktail (Roche, Mannheim, Germany), the remaining homogenates from the brain were immediately frozen at -80 ºC for subsequent cytokine analysis.

Inhibition-ELISA for detection of antigen in the serum and brain

An inhibition-ELISA was performed according to Gomez et al. [17] with some modifications. The diluting buffer used in these experiments consisted of a pool of normal serum or normal brain homogenate that was resuspended at 1:10 in 0.05% PBS-Tween 20 (PBS-Tween). The inhibition plates were blocked by incubation with 200 μL per well of 5% bovine serum albumin (BSA) in PBS-Tween for 2 h at 37 ºC. A standard curve was constructed from 100 μL of different concentrations of *A. kalrae* antigens (from 4 ng to 60 μg/mL) and 100 μL of rabbit anti-*A. kalrae* IgG (20 μg/mL) in diluting buffer. The samples (serum and brain homogenate) were used 1:3, and they were also added to the rabbit anti-*A. kalrae* IgG (20 μg/mL) in diluting buffer. The plates were mixed in a shaker for 30 min at room temperature, and then they were incubated overnight at 4 ºC. The reaction plates (Nunc Maxisorp) were coated with 5 μg/mL CFA in carbonate-bicarbonate buffer at pH 9.6 for 30 min at room temperature and overnight at 4 ºC. The plates were washed 3 times with PBS-Tween and blocked with 1% BSA in PBS-Tween for 1 h at 37 ºC. After the washes, 100 μL from each well of the inhibition plate (standards and samples) were transferred to their respective wells in the reaction plate and incubated for 2 h at 37 ºC. The plates were washed and incubated with a monoclonal anti-rabbit IgG peroxidase (Sigma, St. Louis, MO, USA) at 1:4000 for 1.5 h at 37 ºC. After further washes, the development of reaction was performed with a substrate solution (5 mg ortho-phenylenediamine, 10 mL 0.1 M citrate buffer, pH 4.5 and

10 μL H_2O_2), and the reaction was stopped with 4 N H_2SO_4. The absorbance was read at 492 nm (Labsystems, Helsinki, Finland).

Histopathology

Brains were removed and fixed in 10% buffered formalin. They were then prepared by routine techniques for paraffin embedding. Histological sections were stained using standard protocols with hematoxylin/eosin (HE) for general orientation and Grocott's methenamine silver nitrate technique to detect mycotic structures. The slides were evaluated by light microscopy.

ELISA for total anti-A. kalrae IgG and IgG subclasses

For total anti-*A. kalrae* IgG, ELISA immunoplates were coated with CFA (25 μg/mL), in carbonate-bicarbonate buffer at pH 9.6 for 1 h at 35 ºC and overnight at 4 ºC. The plates were washed 4 times with PBS containing 0.05% Tween 20 and 0.5% skim milk (wash buffer) and blocked with PBS containing 0.05% Tween 20 and 5% skim milk (blocking buffer) for 1 h at room temperature, followed by washing again. The plates were then incubated with 1:20 diluted serum samples for 2 h at 35 ºC. After the washes, goat anti-mouse IgG labeled with peroxidase (Sigma, St. Louis, MO, USA) and diluted 1:4000 was added and incubated for 1.5 h at 35 ºC. The reaction was developed with a substrate solution and stopped with 4N H_2SO_4. The absorbance was read at 492 nm. For IgG subclass analysis, immunoplates were coated with CFA at 5 μg/mL, followed by incubation with serum samples diluted 1:10 and incubated with a rabbit anti-mouse IgG subclass (IgG1, IgG2a and IgG2b) at 5 μg/mL (Zymed Laboratories, San Francisco, CA, USA). The plates were then incubated with monoclonal anti-rabbit IgG peroxidase, diluted 1:4000 (Sigma, St. Louis, MO, USA), and the reaction was developed. The incubation conditions and buffers were the same as previously described.

Delayed-type hypersensitivity (DTH) assay

The DTH reaction was evaluated by using a footpad test. The mice were inoculated in the left hind footpad with 50 μL of sterile CFA (150 μg/mL), and in the right hind footpad with sterile PBS. Footpad thickness was measured with calipers almost 24 h after inoculation, and the difference between the thicknesses of each footpad was calculated.

Circulating immune complexes

For quantification of circulating immune complexes by ELISA, sensitization was performed with rabbit anti-*A. kalrae* IgG at 20 μg/mL. Serum samples were diluted 1:20 and added to plates. Then, the plates were incubated with goat anti-mouse IgG labeled with peroxidase (Sigma, St. Louis, MO, USA) and diluted 1:4000. After developing the assay, the absorbance was read at 492 nm. The incubation conditions and buffers were the same as previously described.

Cytokine analysis

IFN-γ, IL-17, IL-4 and IL-10 levels in serum and in brain and kidney homogenates were quantified with ELISA kits (eBioscience, Inc., San Diego, CA, USA), and the concentrations were determined by the provided standard curves, according to the manufacturer's instructions.

Statistical Analysis

The data were analyzed by GraphPad Prism 6.0 software (GraphPad Software, Inc, La Jolla, CA, USA) by using ANOVA and Tukey's test or t-test; we considered $p<0.05$ to be statistically significant. The correlation (Pearson) was defined as a moderate correlation when $0.50 \geq r < 0.75$ and a strong correlation when $r \geq 0.75$.

Results

Neurological syndrome and weight loss

After infection, mice developed complex neurological syndrome, including head tilt, circling and twirling when raised by the tail. Signs began to appear 3 days post infection (p.i.) and new cases continued to appear until day 11 p.i. (Figure 1). Sixty nine percent of the mice presented some type of sign. One mouse (from a total of 29) died between day 6 and 7 p.i. Weight loss was observed in all groups of animals infected with *A. kalrae*. The infected groups lost between 14 to 18% in weight, compared to the control group (Figure 2), even after 56 days of infection.

CFU results

The CFU results from the spleen, liver, kidney, lung, heart and brain are shown in Figure 3. The organism could be recovered from all organs at 7 days p.i., although not all of the lungs were positive. CFU counts were observed to be higher in the spleen, brain and liver. At 14 days p.i., cultures were primarily positive in the spleen, kidney and liver. At 28 days p.i., the organism could be recovered from every organ except the lung. The fungus continued to be viable even after 56 days of infection.

Antigen level

Antigen levels were analyzed in the serum and brain. Antigenemia was demonstrated to be high during all of the investigated periods of infection. In the brain, the antigen level was increased throughout the experiment, with the highest level at 7 days p.i. (Figure 4).

Histopathology

In the histological analysis of brain sections stained with HE, alterations were observed in the cerebral cortex, which consisted mainly of inflammatory reactions, with occasional vasculitis and gliosis. Inflamatory infiltrates were observed in most animals at day 7 p.i.. The number of animals with tissue

reactions decreased over the period of infection, and the scarring process could be observed in a few animals. In Grocott-stained slides, it was possible to detect the fungus, mainly at 7 days, and at 14 days on very few slides.

Total specific IgG levels

There was a gradual increase in specific IgG levels over the course of infection (Figure 6).

DTH assay

Infected mice developed cutaneous reactivity to *A. kalrae* antigens at 14 days p.i. The DTH response continued 28 and 56 days p.i., even though there was no difference compared to the control group at these time points (Figure 7).

Immune complexes levels

There was immune complexes (IgG-*A. kalrae* antigen) formation at days 14 and 28 p.i., with a decrease occurring 56 days p.i. (Figure 8).

Cytokine analysis

In the cytokine analysis, there was a significant decrease in serum IFN-γ levels 14 days p.i. IL-4 levels were increased 7 and 56 days p.i., and IL-17 levels increased at day 7 and decreased at 56 days p.i. (Figure 9). In brain homogenates of infected mice, there was a decrease in these cytokine levels at 56 days p.i. (Figure 10).

Specific IgG subclasses

The IgG subclasses were predominantly composed of IgG1, followed by IgG2a and IgG2b, at all days investigated in the infected mice (Figure 11).

Correlation test

There was a strong correlation between CFU counts and antigen levels in the brain (r= 0.89) and moderate negative correlation between brain antigen and IgG levels (r= -0.72).

Discussion

As has been previously described in the literature, behavioral changes were observed in mice infected with the fungus *A. kalrae* in this study. The mice developed neurological syndrome in the initial phase of infection that consisted of head tilt, circling and twirling when raised by the tail, as described by Tewari and Macpherson (1968) [13]. In the early phase of infection, several organs contained fungi, possibly due to the route of inoculation utilized (i.v.). Although the fungus is considered to be an opportunistic agent, in this work, infected animals experienced a significant amount of weight loss over the course of infection, suggesting this microorganism has the potential to be a pathogen, even in immunocompetent hosts.

Higher CFU counts were found in the spleen, liver, brain and kidney. Despite the initial CFU levels in the brain, more rapid clearance of the microorganism was observed in this organ. However, there were viable fungi detected up to 28 days after infection. The intensity of infection was also assessed using capture ELISAs with both brain tissue and blood samples. There was a strong correlation between CFU counts and antigen levels in the brain. A significant increase in *A. kalrae* soluble antigen levels was observed both in the brain and in the circulation, and a level higher than the control was also maintained through the end of the evaluation period.

Histological analysis showed inflammatory foci in the brains of infected animals, mainly at the beginning of infection. The inflammatory response may have decreased after clearance of the fungi in the brain. Swenberg et al. (1969) previously demonstrated that the cellular response was higher during the first 3 days and then decreased, and there were no lesions present in the brain after 7 days, or the lesions were small focal infiltrations that were later characterized by repair and scarring [15]. Thus, our results are comparable to the findings of

other studies with this fungus. However, we could see reactions to the fungus in tissues even after 56 days of infection. With Grocott staining it was possible to identify fungal hyphae, which were higher in number at 7 days p.i.

Over the course of infection, induction of the immune response occurred, resulting in decreased levels of infection, which was observed via CFU counts. Here we observed an expected induction of cellular and humoral immune responses. There was a progressive increase in the level of IgG. However, the DTH response unexpectedly decreased during the course of infection, suggesting that the fungus *A. kalrae* can induce suppression of cellular response. This observation may be the reason that the fungal infection was not completely eliminated from various organs and why soluble antigen levels remained higher in the bloodstream and in the brain.

Immunossuppression has been detected in paracoccidioidomycosis (PCM), the systemic mycosis caused by *Paracoccidioides brasiliensis*, due to some soluble antigens such as gp43 and gp70 [18]. In this type of mycosis, it has been suggested that depression of the T cell response is correlated to the amount of circulating immune complexes. These immune complexes, which are composed of high affinity antibodies bound to fungus components, may be involved in the genesis of cell-mediated immunity in PCM patients [19-21]. Analyzing the correlation between immune complexes and DTH in our study, a moderate negative correlation was only observed at day 14, and a low level of immune complexes was observed at a later period. Therefore, in the case of infection by this fungus, immune complexes are most likely not very relevant for the suppression of cellular response.

In several fungal infections models, the Th1 cellular response is the major defense mechanism [22, 23]. In the present study, we evaluated the systemic levels of Th1 (IFN-γ) and Th2 (IL-4 and IL-10) cytokines, and also the IgG subclasses associated with these patterns. Decreased IFN-γ levels are associated with increased IL-4 production, which inhibits the Th1 response [24]. In this study, a decrease of IFN-γ (14 to 56 days) and an increase in IL-4 levels (7 and 56 days) was detected, suggesting that the Th2 response developed over the course of infection. This pattern of immune response may be responsible for the decreased DTH response observed here. In agreement with

the Th2 cytokines, higher levels of IgG1, which are correlated with a Th2 response, were observed over the course of the infection. The Th2 pattern is generally harmful to the host in fungal infections [22], and the dissemination of the fungus is associated with high levels of specific antibodies, as has been described in experimental PCM [25]. In this study, higher specific IgG levels during the course of infection and a negative Pearson correlation were also observed. The IgG1 and IgG2a subclasses can affect opsonization and increase the phagocytosis of microorganisms [26, 27]. However, the participation of Th1 lymphocytes is needed for optimal action of phagocytic cells [28].

There is no data on the immune response to *A. kalrae* infection, but it is possible that the Th1 response is more relevant than IgG1 because higher levels of these antibodies were detected primarily 56 days after infection. And these antibody levels were concurrent with the continuous cachexia and antigenemia observed in the infected animals.

Considering the importance of the brain investigation because of the behavioral change and more rapid clearance of fungal burden in the brain compared to liver and spleen, cytokine levels were investigated in this organ. At 56 days after infection, decreased levels of both cytokine patterns (Th1=IFN-γ, Th2= IL-4 and IL-10) were detected. It is unclear whether the no Th2 polarization was beneficial for the brain; therefore, this effect warrants further study.

Th17 cells/IL-17 play an important role in the protective defense against fungal infections, and this branch of immunity has been demonstrated to be fundamental in modulating the fungal burden in experimental infections, including *Candida albicans*, *Pneumocystis carinii* and *Cryptococcus neoformans* [29, 30]. In our infection with *A. kalrae*, serum IL-17 was elevated 7 days after infection, and levels gradually decreased after that point, which may explain the persistence of viable fungi in various organs over the course of infection. In the brain there was no increase in IL-17 in the infected group compared to the uninfected group. Additionally, 56 days after infection there was a decrease in levels of this cytokine. However, even in the control group, levels of this cytokine were higher in the brain than in serum, which could benefit this organ. According to the literature, cytokines that act within the central nervous system

can originate from a number of sources, including neurons, astrocytes, brain endothelial cells, microglia, and immune cells. Immune cells can enter the central nervous system (CNS) from the periphery. Additionally, many cytokines can be transported across the blood-brain barrier [31].

Considering that there was no significant alteration in cytokine levels in the brain during the early stage of assessment, the behavioral changes that were observed during this period were not due to the cytokines that were evaluated. Instead, the changes were possibly attributable to direct action by the fungus, which was present in large quantities during this period. However, as a greater inflammatory response was observed during this time, it will be important to study additional proinflammatory cytokines, chemokines and other factors.

This work demonstrates the first evidence of systemic and cerebral immunomodulation over the course of experimental mouse infection with *A. kalrae*.

Conflict of interest statement:

The authors have no financial conflict of interest.

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Figures

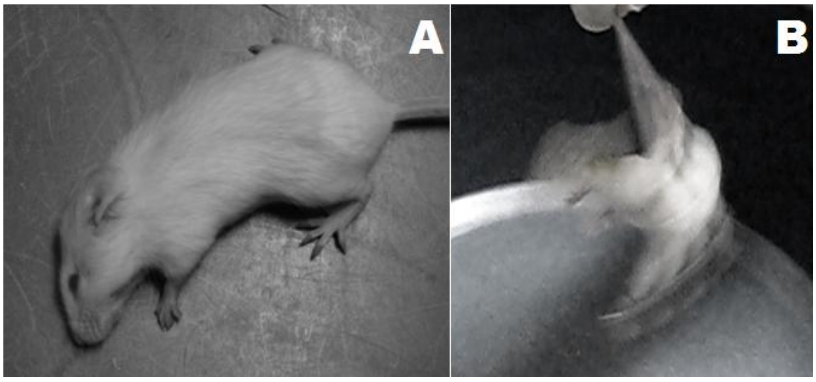

Figure 1. Neurological signs in infected mice. The mice were infected with $5x10^6$ cells of *A. kalrae* and monitored daily for behavioral alterations. The signs, which included head tilt (A) and twirling (B), began to appear 3 days after infection.

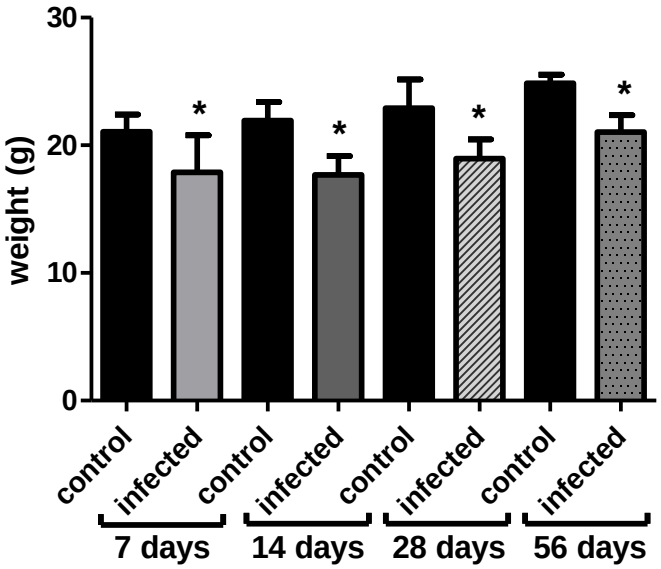

Figure 2. Weight loss in infected mice. The mice were infected with $5x10^6$ cells of *A. kalrae* and weighed after 7, 14, 28 and 56 days of infection. * $p<0.05$ compared to control.

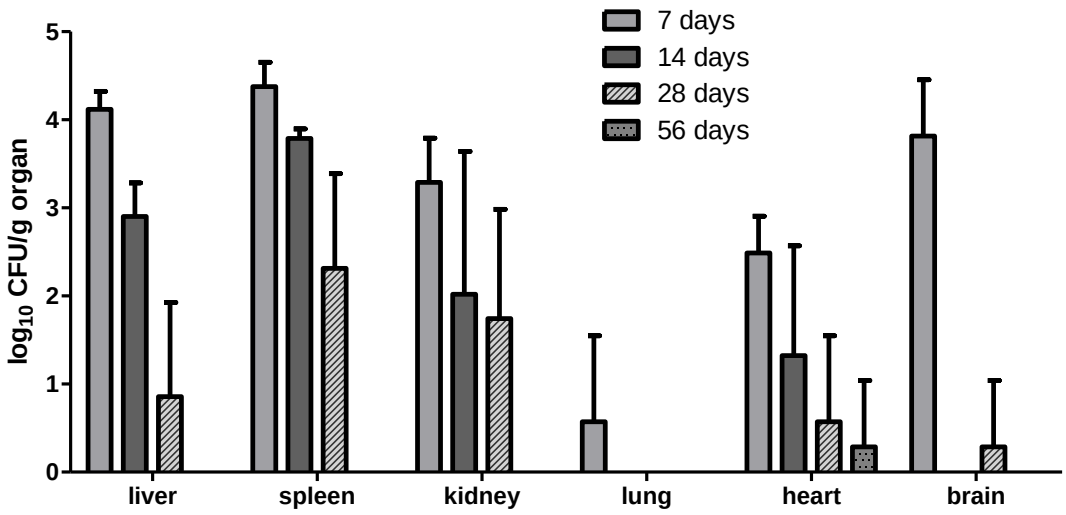

Figure 3. *A. kalrae* CFU determination in the organs of infected mice. During experimental infection with *A. kalrae* at days 7, 14, 28 and 56 p.i., organs homogenates (0.2 g tissue/mL) were plated on PDA and colonies counted after 48 h of incubation at 35 ^{o}C.

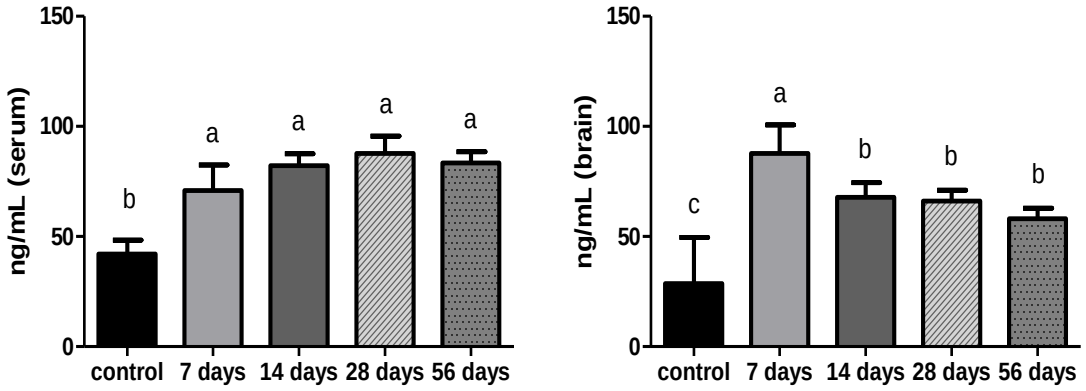

Figure 4. Antigen levels in the serum and brain. Antigen levels (in ng/mL) in serum and brain homogenate (0.2 g/mL) samples were measured by inhibition-ELISA in infected mice (7, 14, 28 and 56 days after infection) and controls not infected.

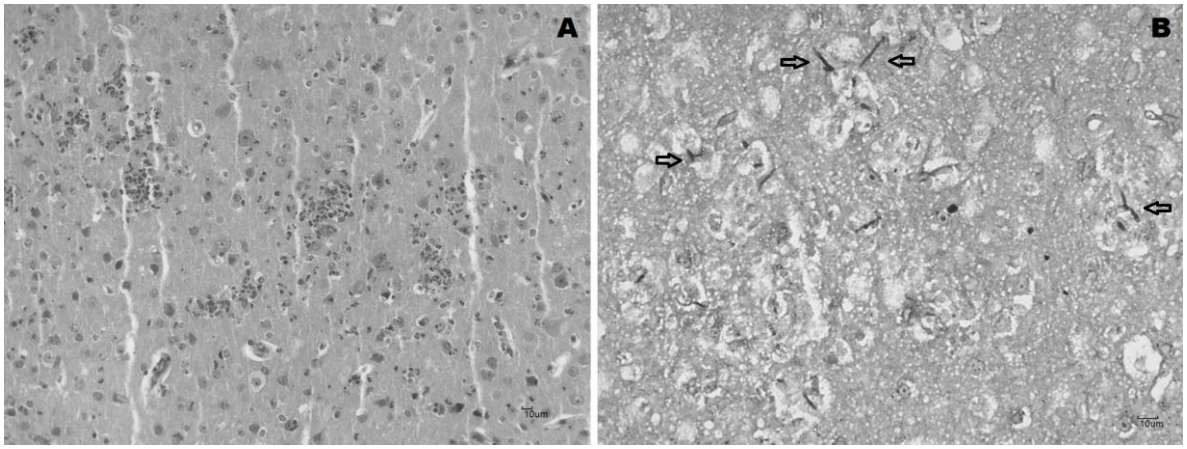

Figure 5. Histopathological analysis. A section of a brain from an infected animal 7 days after infection, which shows inflammatory infiltrates stained with HE (A) and fungal hyphae, indicated with arrows, stained with Grocott's methenamine silver (B).

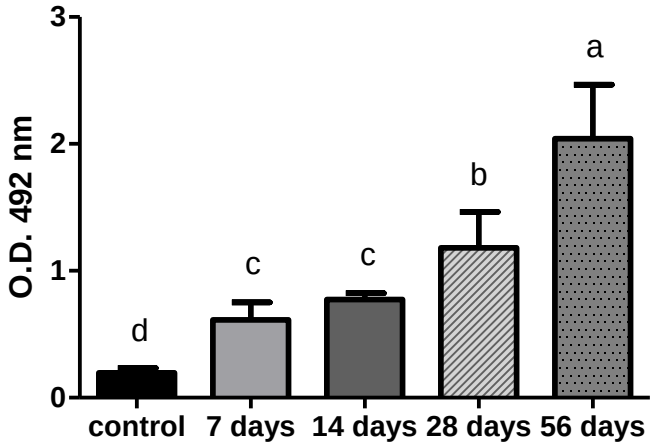

Figure 6. Serum anti-*A. kalrae* IgG levels determination. Serum samples of infected mice with *A. kalrae* and controls not infected were analyzed by indirect ELISA for specific IgG. O.D., optical density.

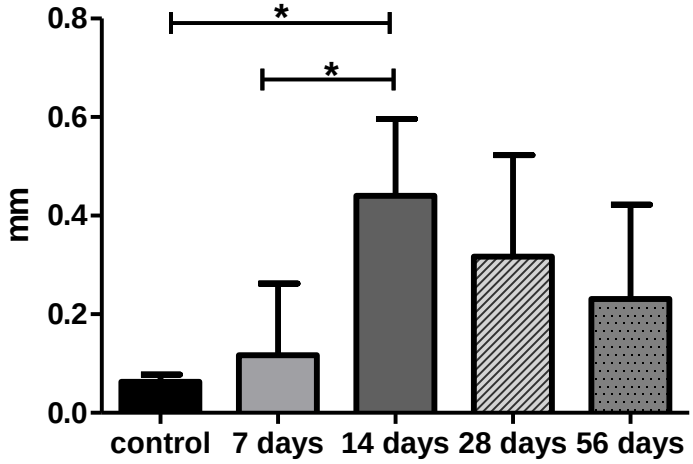

Figure 7. DTH test with total soluble *A. kalrae* antigens. The reaction was evaluated employing a footpad test. The mice were inoculated with CFA (or PBS as control), and footpad thickness was measured almost 24 h later. The graph shows, in mm, the difference between CFA and PBS. *p<0.05.

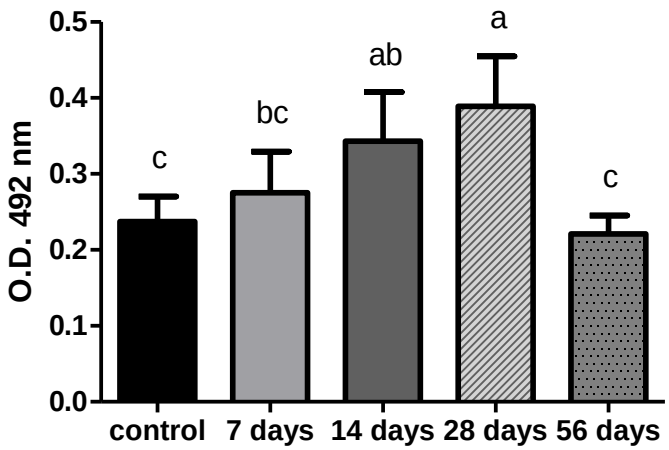

Figure 8. Serum immune complexes levels determination. The serum immune complexes (IgG-antigen) from different days after infection were detected by ELISA. O.D., optical density.

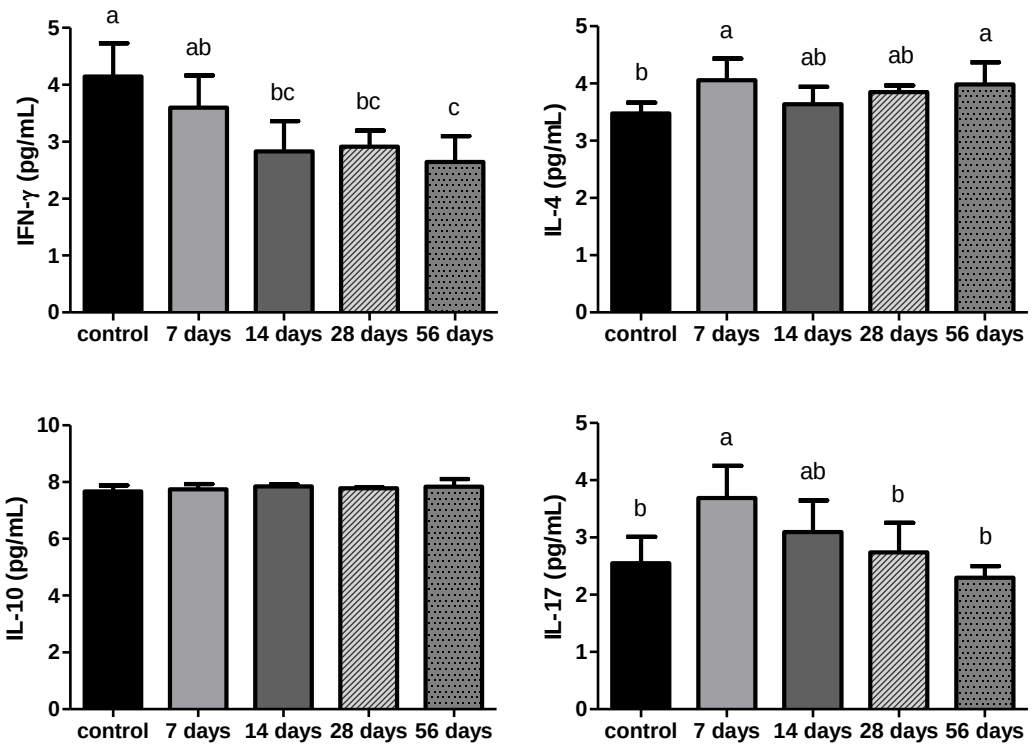

Figure 9. Cytokines levels in serum of infected mice. IFN-γ, IL-4, IL-10 and IL-17 were measured in serum from mice infected with *A. kalrae* and from the control group at 7, 14, 28 and 56 days after infection. The levels are shown in pg/mL, and they were calculated based on a standard curve.

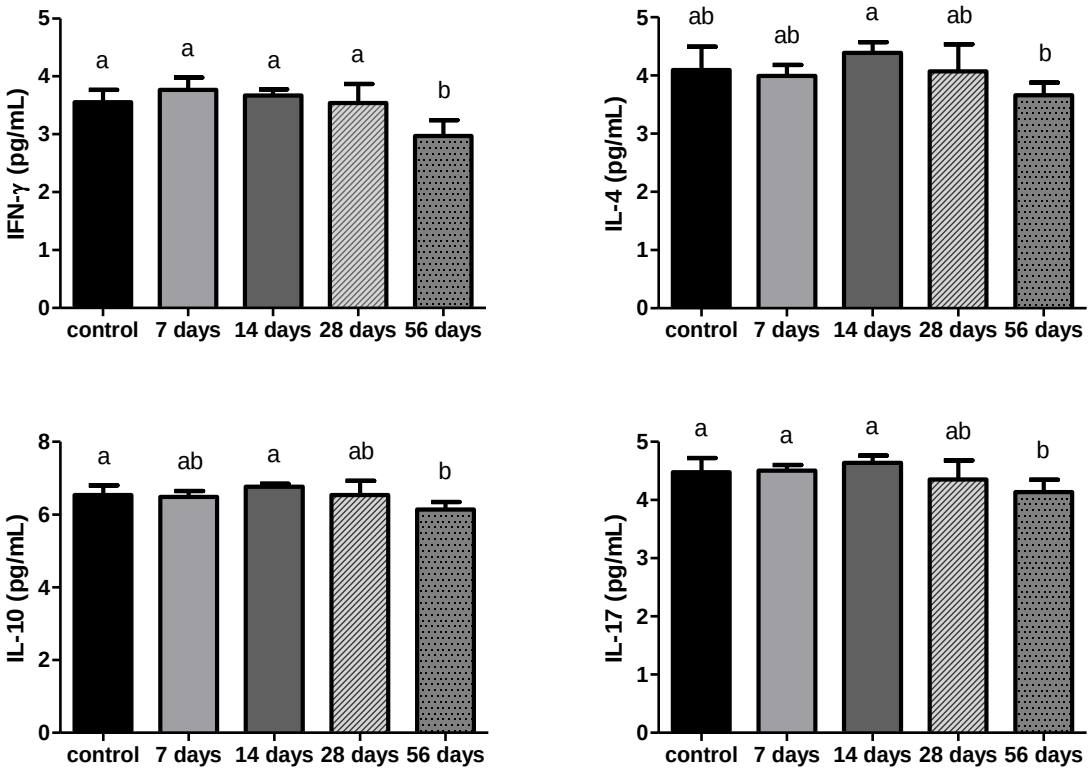

Figure 10. Cytokines levels in the brains of infected mice. IFN-γ, IL-4, IL-10 and IL-17 were measured in brain homogenates from mice infected with *A. kalrae* and from control group at 7, 14, 28 and 56 days after infection. The levels are shown in pg/mL, and they were calculated based on a standard curve.

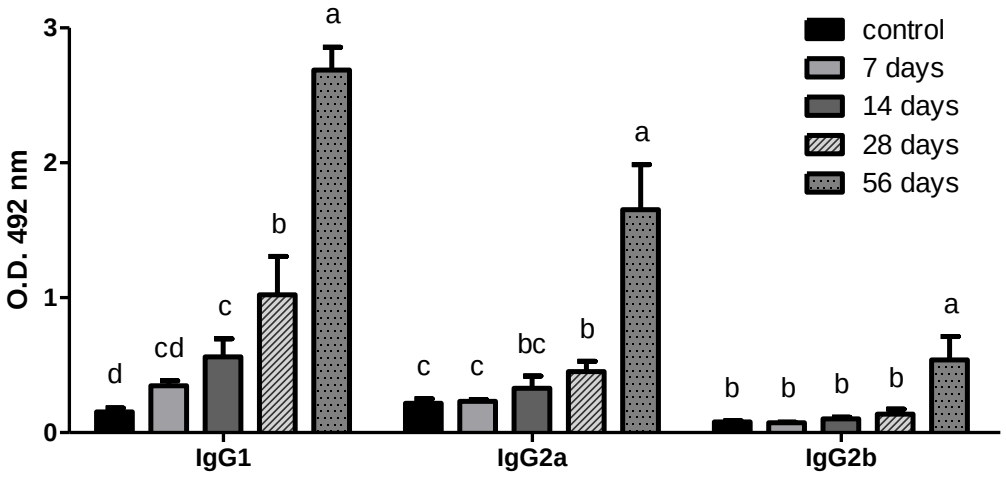

Figure 11. Serum anti-*A. kalrae* antigens IgG subclasses levels. Serum samples of infected mice with *A. kalrae* and controls were analyzed by ELISA on different days p.i. The letters indicating significance belong to the same subclass group. O.D., optical density.

7. CONCLUSÕES GERAIS

Arthrographis kalrae produz componentes solúveis com atividade hemolítica e citotóxica.

Os fatores hemolíticos solúveis de *A. kalrae* são termoestáveis e possivelmente glicoproteína(s) de alta MM.

Os componentes citotóxicos parecem ser de baixa MM, sendo possivelmente, portanto, fatores distintos dos que causam hemólise.

O processo de sensibilização de hemácias com antígenos solúveis permite a obtenção de anticorpos com capacidade de inibir hemólise.

A infecção de camundongos com o fungo *A. kalrae* induz síndrome neurológica e perda de peso.

A infecção de camundongos com *A. kalrae* por via endovenosa induz disseminação do fungo em vários órgãos e em maior concentração no baço, fígado e cérebro, sugerindo maior afinidade do fungo a estes órgãos.

No decorrer de infecção experimental murina com *A. kalrae* ocorre diminuição da carga fúngica, sugerindo o potencial de controle de infecção pelo camundongo Balb/c.

No decorrer da infecção experimental murina com *A. kalrae*, ocorre aumento gradual de IgG específica com predominância da subclasse de IgG1.

A infecção de camundongos com o fungo *A. kalrae* induz resposta de DTH, sugerindo indução de resposta imune celular, todavia no decorrer da infecção ocorre diminuição da resposta DTH, sugerindo indução de imunossupressão celular.

No decorrer da infecção experimental murina com *A. kalrae*, ocorre diminuição gradativa de IFN-γ e aumento de IL-4 sérica, sugerindo modulação de resposta de padrão Th2.

No cérebro ocorre também modulação nos níveis de citocinas com diminuição de IFN-γ, IL-4 e IL-10, todavia essa modulação ocorre na fase mais tardia de infecção experimental murina com *A. kalrae*.

Tanto o nível sistêmico como cerebral da citocina IL-17 encontra-se diminuída na fase mais tardia, sugerindo a modulação dessa citocina tanto em nível sistêmico como local.