Lymphoproliferation and cytokine profiles in human peripheral blood mononuclear cells stimulated by Cryptococcus neoformans

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Cell-mediated immunity is critical to host defenses against the fungal infection cryptococcosis. Here, two functions critical to effective cell-mediated immunity (CMI), lymphoproliferation and cytokine release, were studied in Cryptococcus neoformans-stimulated peripheral blood mononuclear cells (PBMC) from seven healthy donors (controls) and two patients with cryptococcosis. PBMC responses to C. neoformans were compared with responses to Candida albicans. Control and patient PBMC had significant lymphoproliferation in response to whole C. neoformans, with peak proliferation seen following 8 days of culture, but only patient PBMC proliferated when stimulated with C. neoformans mannoprotein. C. neoformans-stimulated control PBMC released IL-2, IFN-γ, and IL-10 into the supernatant with peak or near peak concentrations of these three cytokines generally seen by day 1. Release of IL-4 was low or undetectable. In contrast, C. neoformans-stimulated patient PBMC released IFN-γ, which peaked on day 7, as well as IL-4, IL-10, and in one of two patients, IL-2. Cytokine release occurred later in patient (compared with control) PBMC. Lymphoproliferation and cytokine release were similar comparing control PBMC stimulated with C. neoformans versus Candida albicans. Thus, the magnitude and kinetics of the lymphoproliferative response to whole C. neoformans is similar comparing PBMC from controls and patients, but the cytokine profiles differ. Moreover, the capacity of patient PBMC to respond to soluble mannoprotein lends support to studies of mannoprotein components as vaccine candidates.

Keywords C. neoformans, human PBMC, profiles

Introduction
In the past decade, it has become apparent that for many infectious agents, the nature of the specific immune response is determined by the set of cytokines produced by CD4+ T helper (Th) cells [1–3]. Th1 cells secrete IFN-γ and IL-2 which drive the cell-mediated immunity (CMI) response by activating T cells and stimulating phagocyte-mediated host defenses. Th2 cells secrete IL-4 and IL-10 which inhibit macrophage functions. IL-4 also stimulates B cell antibody production. A fine-tuned Th1 response appears necessary for successful resolution of infection: an overexuberant Th1-type response can lead to damage to host cells from the inflammatory response whereas an inappropriate Th2-type response can result in ongoing infection.

Compared with persons in the general population, the prevalence of infections due to the fungus C. neoformans is greatly increased in individuals with impaired CMI, particularly persons with AIDS. Following in vitro incubation of PBMC obtained from healthy human donors with heat-killed C. neoformans, lymphoproliferation occurs, with peak responses seen after approximately 7–11 days of culture [4–6]. C. neoformans has a worldwide distribution and exposure to the fungus is presumably...
common [7]. Nevertheless, it is unknown whether lympho-
proliferation stimulated by whole C. neoformans is in fact
an amnestic response to past exposure to C. neoformans
or a consequence of past exposure to other micro-
organisms possessing epitopes which cross-react with
cryptococcal antigen(s). Evidence for the latter is the
finding that PBMC from persons who have recovered
from cryptococcosis respond to both whole C. neoformans
and a soluble cryptococcal culture filtrate whereas PBMC
from healthy donors respond to whole organisms only [4].

In the experiments reported herein, we further defined
the conditions under which human PBMC from normal
donors and from patients with cryptococcosis proliferate
in response to whole C. neoformans and a soluble manno-
protein fraction derived from C. neoformans. Moreover,
we examined the cytokine profile stimulated during
the lymphoproliferative response to C. neoformans by
measuring release of IFN-γ, IL-2, IL-4 and IL-10. Finally,
we compared the nature of lymphoproliferative and
cytokine response to C. neoformans with that of another
medically important fungus, C. albicans.

Materials and methods

Materials

All reagents were obtained from Sigma Chemical Co.
(St Louis, MO) unless stated otherwise. All experiments
were performed under conditions carefully designed to
minimize endotoxin contamination as described [8,9].
RPMI 1640 and PBS were obtained from Biowhittaker
(Walkersville, MD) and contained less than 0.005 endo-
toxin units per ml. Pooled human serum (PHS) was
prepared by combining serum from greater than ten
healthy donors under conditions carefully designed to
minimize endotoxin contamination and preserve com-
plement activity. Heat-inactivated PHS (H-I-PHS) was
prepared by heating PHS to 56 °C for 30 min. Unless
otherwise indicated, medium was RPMI 1640 contain-
ing 10% PHS, and all incubations were performed in
humidified air supplemented with 5% CO₂ at 37 °C.

Fungi

Serotype A strain 145 [9-11] of C. neoformans was grown
on Sabouraud glucose agar at 25 °C for 4 days. Under
such conditions, capsule thickness averaged 1.2 μm [9].
Where indicated, C. neoformans was grown on asparagine
agar or in RPMI 1640 in the absence of bicarbonate, pH
6.0, as described [9]. A previously characterized isolate of
C. albicans [12,13] was grown in the yeast phase on
Sabouraud glucose agar at 25 °C for 4 days. Prior to use,
fungi were heat-killed at 50 °C for 30 min, washed at least
five times in PHS, and stored at 4 °C. Overgrowth of
cultures during the incubations precluded uses of live
fungi. Fungi were free of significant amounts of endotoxin
as determined by the inability of the LPS antagonist
Rhodobacter sphaeroides lipid A (a gift of Dr Nilo
Qureshi, Middleton VA Hospital, Madison, WI) to
inhibit TNFa release from fungal-stimulated PBMC [8,9].

C. neoformans mannoprotein

Mannoprotein derived from C. neoformans acapsular
strain cap67 (a generous gift of Dr Robert Cherniak,
Georgia State University, Atlanta, GA) was purified as
described [14,15]. Briefly, an ultrafiltrate from a culture
of cap67 was applied to a column of conconavalin A–
Sepharose 4B. The mannooprotein fraction corresponds
to the peak eluted with 0.2 M methyl-α-D-mannopyranoside
and consists of 21% protein. Mannoprotein was dialysed,
desalted, dissolved in PBS and stored in aliquots at
–70 °C until use. This fraction was used at a final
concentration of 25 μg ml⁻¹.

Peripheral blood mononuclear cells (PBMC)

Human peripheral blood was obtained by venipuncture
from normal volunteers recruited through advertisements
placed in the medical center (controls) or persons with
cryptococcosis (patients). Blood was anticoagulated with
heparin and the PBMC purified by centrifugation on a
Ficoll–Hypaque density gradient [9].

Lymphocyte proliferation

Lymphocytes (1 × 10⁵) and fungal cells (1 × 10⁵) were
co-incubated in 96-well flat-bottom plates (Corning Glass
Works, Corning, NY) containing a final volume of 150 μl
of medium per cell well [5]. Wells were pulsed with
[³H]-thymidine (0.5 μCi; Dupont NEN, Boston, MA)
during the last 18 h of culture. Plates were harvested after
4, 8 and 11 days of culture with a cell harvester (PHD;
Cambridge Technology, Watertown, MA) onto glass filter
paper and counted by scintillation spectroscopy. All pro-
liferation assays included negative controls containing
PBMC but no fungi and positive controls containing
PBMC plus 1 μg of phytohaemagglutinin (PHA) per ml.
All donors had vigorous proliferative responses to PHA,
with peak responses seen on day 4 (data not shown).

Cytokine release

Lymphocytes (1 × 10⁶) and fungal cells (1 × 10⁶) were
co-incubated in 24-well flat-bottom plates (Costar;
Cambridge, MA) containing a final volume of 1 ml of
medium per cell well [5]. Wells containing PBMC alone

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also were included. After 1, 3 and 7 days of incubation, supernatants were collected and frozen at -70% until cytokine assay.

Cytokine concentrations in cell supernatants were quantitated by ELISA using antibody pairs and standards purchased from Genzyme Corporation (Cambridge, MA) for IFN-γ and PharMingen (San Diego, CA) for IL-4 and IL-10 according to the manufacturer’s directions. IL-2 was measured with a commercial kit (BioSource International, Camarillo, CA). All ELISAs were sensitive to 10 pg ml⁻¹.

**Statistics**

Means and SE were compared using the two-tailed, two sample t-test on a statistical software program (SigmaStat for Windows, Jandel Scientific Software, San Rafael, CA).

**Results**

**Optimal conditions for lymphoproliferation**

Initial experiments defined the optimal conditions for lymphoproliferation of control PBMC in response to heat-killed *C. neoformans* and *C. albicans*. Consistent with previous data [6], optimal values were found using an equal number (10⁵) of fungi and PBMC per well (data not shown). Both the kinetics and the magnitude of the lymphoproliferative response were similar when comparing autologous serum with PHS (data not shown). Consistent with the known role of complement in monocyte recognition of *C. neoformans* [16], heat-inactivation of PHS inhibited the lymphoproliferative response to *C. neoformans* from five donors by a mean of 67% (range = 39–80%; P < 0.001). Lymphoproliferation in response to *C. neoformans* was similar comparing organisms grown on Sabouraud glucose agar, asparagine agar and RPMI 1640 without bicarbonate (data not shown). Subsequent experiments used fungi grown on Sabouraud glucose agar and opsonized with PHS.

**Lymphoproliferation and cytokine profiles in fungal-stimulated normal PBMC**

Lymphoproliferation and cytokine release were measured simultaneously in PBMC from seven healthy donors stimulated with *C. neoformans* and *C. albicans*. As expected based on previously published data [4-6,17], the lymphoproliferative response to both stimuli peaked around day 8 (Fig. 1). Compared with unstimulated cells, PBMC from all seven donors had a significant (P < 0.01) response to both fungi, with peak proliferation ranging from 3865 to 60 753 cpm for *C. neoformans* and 5755 to 50 682 for *C. albicans*. PBMC from none of five donors significantly proliferated when stimulated with a soluble mannoprotein fraction derived from acapsular strain cap67 (mean cpm 1939, 1040 and 1096 on days 4, 8 and 11, respectively, with MP fraction compared with 2404, 819 and 1878 with PBMC alone).

Following stimulation of PBMC from control subjects with whole *C. neoformans* and *C. albicans*, IL-2, IFN-γ, and IL-10 were released into the supernatant (Figs 2 and 3). Although peak lymphoproliferation was not seen until day 8, peak or near peak concentrations of these three cytokines were generally seen by day 1. There was a trend in favour of increased IFN-γ concentrations in PBMC stimulated with *C. albicans* compared with *C. neoformans*, although this did not reach statistical significance (P = 0.057). In contrast, the two fungi stimulated similar amounts of IL-2 and IL-10 release from PBMC. Concentrations of IL-4 were below the limits of detection in PBMC from 4 of the 7 donors stimulated with either *C. albicans* or *C. neoformans*. Moreover, when IL-4 release was detected, it was transient and at levels < 100 pg ml⁻¹. Median values for IL-2, IL-4, IFN-γ, and IL-10 were below the limits of detection (< 10 pg ml⁻¹) in supernatants from PBMC left unstimulated. Additional release of any of the four cytokines was not detected when measured at day 10 of culture (data not shown).

**Lymphoproliferation and cytokine release in patients with cryptococcosis**

In the final experiment, two patients with cryptococcosis without known immunosuppression (including negative serologies for HIV) were studied. The first patient was a 24-year-old man who presented with a 1 year history of
fevers, cough, weight loss and a cavitary lesion on chest radiographs which failed to respond to antituberculosis and antibacterial therapy. He had a history of heavy exposure to aerosolized pigeon droppings. He was diagnosed with pulmonary cryptococcosis following a lung biopsy. Evaluation for extrapulmonary disease including meningitis was negative. This patient was on oral fluconazole when studied. The second patient was a 65-year-old man without significant past medical history who presented with behavioural changes and syncope. He raised ducks and other birds as a hobby. Work-up revealed cryptococcal meningitis complicated by multiple cerebral and cerebellar cryptococcomas. This patient was receiving amphotericin B and 5-flucytosine when studied. Immunophenotyping of lymphocytes from patients 1 and 2 were completely within normal limits, including CD4 counts of 824 and 676 cells mm\(^{-3}\).

PBMC from both patients responded to whole C. neoformans and the mannoprotein fraction derived from a culture filtrate of an acapsular strain (Fig. 4). Patient PBMC released modest amounts of IFN-\(\gamma\), which progressively increased over the time course studied, when stimulated with whole heat-killed C. neoformans (Fig. 5). IL-2 release was detected in only one of the subjects. IL-4 release was seen in PBMC from both patients. Moreover, peak levels of IL-4 were higher than those seen in supernatants from control PBMC stimulated with C. neoformans (as demonstrated on Fig. 2). Patient PBMC also released IL-10 when stimulated with C. neoformans.

Patient PBMC were also tested for lymphoproliferation and cytokine release following stimulation with whole heat-killed C. albicans. Responses were similar to that seen with control PBMC. Peak lymphoproliferation was seen on day 8 (data not shown). Cytokine release (pg ml\(^{-1}\)) for patient 1 on days 1, 3 and 7 was as follows: IFN-\(\gamma\) release was 1424, 3901 and 3636, respectively; IL-2 was 91, 104 and 115 pg ml\(^{-1}\), respectively; IL-10 was 4095, 1492 and 735 pg ml\(^{-1}\), respectively; and IL-4 was undetectable at three time points. Cytokine release (pg ml\(^{-1}\)) for patient 2 on days 1, 3 and 7 was as follows: IFN-\(\gamma\) release was < 10, 90 and 64, respectively; IL-2 was < 10, < 10 and 17, respectively; and both IL-2 and IL-10 were undetectable at all three time points.

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Discussion

It is presumed that protection against cryptococcosis is dependent, at least in part, upon the development of antigen-reactive T cells [18,19]. However, the exact identity of the cryptococcal antigen(s) responsible for stimulating immunity remains elusive [20]. Hoy et al. demonstrated that T cells from recovered cryptococcosis patients, but not control T cells, proliferate in response to a crude cryptococcal culture filtrate [4]. Our data extend these findings by demonstrating that lymphoproliferation is in response to antigens contained within the mannoprotein fraction of the culture filtrate. Similarly, the mannoprotein fraction elicited strong delayed-type hypersensitivity responses in mice immunized with C. neoformans [14]. Thus, while these results do not preclude other cryptococcal antigens critical for the CMI response, they do suggest an important role for mannoproteins.

The cytokine profile observed in supernatants of control PBMC was similar after stimulation with C. neoformans compared with C. albicans. Overall, the response tended to favour a Th1 type profile, with PBMC from all
but one donor making both IFN-γ and IL-2 following fungal stimulation. Moreover, release of IL-4 was low or undetectable. Significant release of IL-10 was detected although, as discussed below, the IL-10 may have been derived mainly from monocytes rather than lymphocytes.

Other investigators have examined cytokine profiles in immune cells stimulated with *C. neoformans* and *C. albicans*. Murphy found that spleen cells obtained from mice immunized with cryptococcal culture filtrate antigen emulsified in complete Freund adjuvant produced IL-2 and IFN-γ, but not IL-4 or IL-5 [21]. Vecchiarelli et al. determined cytokine levels in supernatants of human T cells co-cultured with *C. neoformans*-laden alveolar macrophages [22]. IL-2, IFN-γ and IL-4 all were detected, although the levels seen averaged less than 100 pg ml⁻¹. Although direct evidence is lacking, it has been hypoth-

ized that many cases of mucosal candidiasis in humans without apparent defects in CMI are secondary to activation of human *Candida*-specific T_H₁ type cells [23]. In experimental models of candidiasis, a T_H₁ type response correlates with protection while a T_H₂ type response favours persistence of the fungus [24–27].

The data obtained with the PBMC from the patients with cryptococcosis are limited somewhat by the small number of subjects studied. Unfortunately, efforts to recruit other patients who also lacked apparent predisposing immune defects were unsuccessful. The vast majority of cryptococcosis patients seen in the 1990s have AIDS or another identifiable immunocompromising condition. Hoy et al. demonstrated that in subjects seropositive for HIV, progression to AIDS is associated with a loss of T cell proliferative responses to *C. neoformans* [28]. Similarly, we demonstrated that PBMC from patients with cryptococcosis and idiopathic CD4⁺ T lymphocytopenia do not proliferate when stimulated with *C. neoformans* [29] (and S. M. Levitz, unpublished results). T and NK cells directly bind to *C. neoformans* and *C. albicans* and, under the proper conditions, mediate fungistasis [30]. Recently, we demonstrated that PBMC extensively depleted of monocytes release IFN-γ (but not IL-4 and IL-10) when directly stimulated by *C. neoformans* and *C. albicans* [8]. Moreover, purified populations of both NK and T cells release IFN-γ following direct fungal stimulation. However, the amount of IFN-γ released in response to fungal stimulation was considerably less than that seen in the studies reported herein using monocyte-replete PBMC. Thus, while some of the IFN-γ released in response to fungal stimulation could be a result of direct stimulation of lymphocytes by the fungi, the bulk of the cytokine released appears dependent on the presence of monocytes.

In addition to lymphocytes, mononuclear phagocytes including peripheral blood monocytes are known to release IL-10. Our data do not address which cell type was responsible for IL-10 secretion when PBMC were stimulated with *C. neoformans* and *C. albicans*. The levels of IL-10 released by PBMC in response to *C. neoformans* were somewhat higher in the present investigation compared with previous studies from our laboratory [31]. While the reason for the disparity remains speculative, it could be related to differences in capsular size [32,33] between the cryptococcal isolates used in the two studies as well as donor-dependent heterogeneity of response.

It is interesting to note that in PBMC from normal donors stimulated with *C. neoformans*, peak release of IFN-γ generally occurred during the first 1–3 days of stimulation whereas peak lymphoproliferation did not occur until day 8. In contrast, when PBMC from the two cryptococcosis patients were stimulated, IFN-γ levels were

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maximal on the seventh day of culture. Moreover, IL-4 was detected at two or more time points in supernatants from patient PBMC (but not control PBMC) stimulated with *C. neoformans*. These data indicate that although the magnitude and kinetics of the lymphoproliferative response to whole *C. neoformans* is similar comparing PBMC from controls and patients, the cytokine profiles differ. Moreover, this observation, combined with the demonstration that only patient PBMC respond to soluble mannoprotein, suggest that a specific CMI response occurs following cryptococcal infection that differs from the response to *C. neoformans* seen in uninfected individuals. Efforts to identify candidate antigens for cryptococcal vaccines probably should focus on antigens recognized by apparently immunocompetent persons who have recovered from cryptococcosis or healthy individuals with a history of heavy exposure to *C. neoformans* [34].

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