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Encapsulation of *Cryptococcus neoformans* regulates fungicidal activity and the antigen presentation process in human alveolar macrophages

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SUMMARY

Our previous studies have shown that unstimulated alveolar macrophages (AM) play a predominant role as antigen-presenting cells in *Cryptococcus neoformans* infections, while the function as effector cells seems to be of minor relevance. The present study focuses on the role of encapsulation of *C. neoformans* on fungicidal activity and the antigen presentation process of AM. Fungicidal activity in unstimulated AM occurs to a higher degree when the acapsular strain is employed, but this is impaired compared with other natural effectors, such as peripheral blood monocytes (PBM) and polymorphonuclear (PMN) cells. Cryptococcus-laden AM also induce a higher proliferative response in autologous $CD4^+$ lymphocytes when the acapsular strain is used compared with encapsulated yeast. The enhanced blastogenic response is, in part, ascribed to an augmented IL-2 production by T cells. In addition, higher levels of interferon-gamma (IFN-γ), but not IL-4, are produced by the responding T cells, when the acapsular strain is used compared with the encapsulated yeast. Moreover, IFN-γ is able to induce fungicidal activity in AM against the encapsulated yeast and augments killing activity of the acapsular strain. This phenomenon is not mediated by nitric oxide production, but is correlated with an enhancement of fungicidal activity of cytoplasmic cationic proteases. We speculate that encapsulation of *C. neoformans* could down-regulate the development of the immune response mediated by *Cryptococcus*-laden AM at lung level.

Keywords *Cryptococcus neoformans* alveolar macrophages respiratory infections

INTRODUCTION

The increasing interest in pulmonary infections caused by opportunistic pathogens is related to an increase in the number of immunocompromised hosts. In fact, infections of the respiratory tract are one of the most frequent causes of morbidity and death in these patients [1,2]. *Cryptococcus neoformans* is one of the fungi capable of inducing pulmonary and systemic infection in the immunocompromised host. It is an encapsulated yeast which causes infection in patients with impaired cell-mediated immunity, particularly in AIDS patients [3]. The initial site of infection is the lung and alveolar macrophages (AM) are the first line of defence. These cells may be involved in the control of infection and dissemination of fungus to other organs. An important role in the defence against *C. neoformans* is played by natural effectors such as natural killer (NK) cells [4,5], neutrophils and monocytes [6] which kill *C. neoformans* in the absence of specific antibodies. The contribution of AM in defence against this fungus is as yet unclear. Growth inhibition of *C. neoformans* has been demonstrated by human AM [7]; however, interferon-gamma (IFN-γ) [8] and other cytokines, such as tumour necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), are able to enhance destructive activity of macrophages [9]. It is conceivable that the encapsulation of the fungus could restrain the clearance by AM. In fact, the major virulence factor ascribed to this yeast is the polysaccharide capsule which exerts an anti-phagocytic action [10–12], impairs fungicidal activity of professional phagocytes [13], and inhibits the development of T cell-mediated immunity by obstructing ingestion of the fungus by antigen-presenting cells (APC) [14]. Moreover, Mody & Syme suggest that the purified polysaccharide capsule suppresses lymphocyte proliferation [15]. In previous papers we demonstrated that unstimulated AM are able to kill *Candida albicans* [16], but not *C. neoformans*, and that AM play an important role as APC in *C. neoformans* infections, inducing brisk proliferation of α/β TCR-bearing T cells [17].

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In the present study we investigated the role of encapsulation of *C. neoformans* in the interaction with human AM. Our findings suggest that the capsule inhibits AM functions, such as fungicidal activity and antigen presentation, by suppressing the Th1 response induced by *Cryptococcus*-laden AM. This phenomenon could favour the progress of infection from the lung to the central nervous system.

MATERIALS AND METHODS

Reagents and media

RPMI 1640 medium and fetal calf serum (FCS) were obtained from Eurobio Laboratories (Paris, France). Triton X100 was obtained from Sigma Chemical Co. (St Louis, MO). Anti-immunoglobulin fluoresceinated isothiocyanate conjugate (FITC anti-IgG; Sigma), anti-human CD3 FITC, anti-human CD4 FITC, and anti-human CD8 FITC conjugates were obtained from Caltag Laboratories Inc. (South San Francisco, CA). Human IFN-γ was provided by Genzyme Corp. (Boston, MA). All media and buffers used in this study contained less than 1 pg endotoxin/ml as detected by the *Limulus amoebocyte* lysate assay.

Preparation and purification of human AM

AM were collected from 15 healthy non-smoking informed volunteers of both sexes (35–65 years of age) as described elsewhere [16]. Briefly, after laryngeal anaesthesia with 2% lidocaine, bronchoalveolar lavage (BAL) was performed through a fibreoptic bronchoscope (Model BF-type 10; Olympus Co., Tokyo, Japan). Three 50-ml aliquots of sterile saline (0·9% NaCl) warmed to 37°C were infused into one of the segments of the right or left lobe and then removed by gentle suction. Lavage fluid specimens were filtered through coarse gauze, and cells were recovered by centrifugation at 500 ***g*** for 8 min at 4°C. The number of cells harvested was approximately $10–15 \times 10^6$. AM evaluated as positive on non-specific esterase staining cells were >90%. Thereafter, the cells were plated in cell culture Petri plates (Nunc Inter Med, Roskilde, Denmark) at a concentration of $2–3 \times 10^6$/ml in RPMI 1640 medium supplemented with 5% FCS and 100 U penicillin/ml and 100 μg of streptomycin/ml (hereafter referred to as cRPMI) and incubated at 37°C in 5% CO_2 for 1 h. The non-adherent cells were removed by washing the dishes three to five times with warm RPMI 1640 medium; adherent cells were carefully removed using a rubber policeman. The latter cells were 95–98% esterase positive, and cell viability was assessed by the trypan blue dye exclusion test (more than 95% viable).

Preparation of peripheral blood monocytes and polymorphonuclear cells

Heparinized venous blood, obtained from donors at the time of BAL, was diluted with cRPMI. Then mononuclear cells were separated by Ficoll–Hypaque density gradient centrifugation [18]. The pellet containing polymorphonuclear (PMN) cells and erythrocytes was diluted with 10 vol of 0·83% ammonium chloride to lyse erythrocytes. The granulocytes were collected by centrifugation, washed twice in cRPMI, counted and adjusted to the desired concentration. Cells at the resulting interface were washed twice in cRPMI and plated into cell culture Petri dishes (Nunc) and incubated for 1 h at a concentration of $2–3 \times 10^6$/ml. The adherent cells (peripheral blood monocytes (PBM)) were recovered and treated as described above. Non-adherent cells were E rosetted as previously described [17]. The cells recovered were T lymphocytes (E^+) >98% $CD3^+$ as evaluated by flow cytometry analysis.

Microorganisms

Two strains of *C. neoformans* were obtained from Dr J. Orendi (Central Bureau Schimmel (CBS) Cultures, Delft, The Netherlands): the *C. neoformans* variant neoformans serotype A encapsulated strain (CBS no. 6995 = NIH 37 (National Institutes of Health, Bethesda MD)) and *C. neoformans* variant neoformans acapsular mutant (CBS no. 7698 = NIH B-4131). The cultures were maintained by serial passage on Sabouraud agar (Bio Merieux, Lione, France) and harvested by suspending a single colony in RPMI 1640, washed twice, counted on a haematocytometer and adjusted to the desired concentration. The source, morphological characteristics and growth conditions of *C. albicans* have been previously described [16]. *Cryptococcus neoformans* (6995 or 7698) strains and *C. albicans* were inactivated by autoclaving.

Killing activity versus C. neoformans *(6995 or 7698) or* C. albicans

Killing activity was evaluated by colony-forming units (CFU) inhibition assay. Briefly, AM, PBM or PMN (1×10^5) in 0·1 ml of suspension per well were incubated in flat-bottomed 96-well microtitre tissue culture plates (Falcon) with *C. albicans* or *C. neoformans* (6995 or 7698) (1×10^4) in 0·1 ml of RPMI plus 10% human serum (HS). HS in our experimental conditions did not affect the viability of *C. albicans* or *C. neoformans* encapsulated or acapsular strain. AM or PBM mixed with *C. albicans* or *C. neoformans* (6995 or 7698) were incubated for 6 h and PMN for 2 h. After incubation at 37°C under 5% CO_2, the plates were vigorously shaken, monolayers were lysed by adding Triton X100 0·1% in distilled water (final concentration in the well 0·01%), and serial dilutions were prepared in distilled water from each well. Plates (triplicate samples) were made by spreading each sample on Sabouraud dextrose agar, and CFU were visually evaluated after 24 h of incubation at 37°C for *Candida* and 72 h at 28°C for *Cryptococcus*. Control cultures consisted of *C. albicans* or *C. neoformans* (6995 or 7698) incubated without effector cells. Killing activity *versus C. albicans* or *C. neoformans* (6995 or 7698) was expressed as the percentage of CFU inhibition, according to the following formula:

$$\% \text{ killing activity} = 100 - \frac{\text{number of CFU from the experimental group}}{\text{number of CFU from control cultures}} \times 100$$

Phagocytosis assay

Phagocytosis assays were performed as previously described [17,19] by addition of live *C. albicans* or *C. neoformans* (6995 or 7698) to AM monolayers cultured in RPMI plus 10% HS on the cover slip at an effector-to-target cell ratio of 1:10. After 2 h incubation at 37°C in 5% CO_2, the excess of microorganisms was removed by extensive washing. Phagocytic activity was evaluated on Giemsa-stained preparations observed by light microscope. A minimum of 200 cells was scored, and any cells containing one or more yeasts were counted as phagocytic.

Lymphocyte proliferation assay

Lymphocyte proliferation assays were assessed as previously described [17]. Briefly, monolayers of AM or PBM (2×10^4) adhered in 96-well flat-bottomed plates were incubated with or without heat-inactivated 2×10^5 *C. neoformans* (6995 or 7698) for 2 h at 37°C, 5% CO_2 in RPMI plus 10% HS, and used throughout as APC. Then the AM or PBM monolayers were washed to remove non-bound microorganisms. Subsequently autologous $T(E^+)$ cells (1×10^5) in RPMI plus 10% HS were added to culture. At various days, cultures were pulsed for 6 h with 0·5 μCi ^{3}H-dThd (Amersham International, Aylesbury, UK), thereafter the cells were collected onto filter paper using a cell harvester (Flow Labs, McLean, VA). The dried filters were counted directly in a β counter (Packard Instruments Inc., Downers Grove, IL). Proliferation was expressed by mean values of indicated replicates ± s.d.

Flow cytometry analysis

Lymphocytes harvested on day 7 were washed twice in PBS containing 0·5% bovine serum albumin (BSA) and 0·1% sodium azide. Cells (1×10^6) in 50 μl were mixed with 10 μl of antibodies FITC anti-IgG or FITC anti-CD3 or FITC anti-CD4 or FITC anti-CD8. After 45 min of incubation the cells were washed three times and analysed using a flow cytometer (Becton Dickinson, FACScan).

Cytokine determination

IL-2, IFN-γ, and IL-4 were determined by human IL-2 ELISA kit, by human IL-4 ELISA kit and human IFN-γ ELISA kit obtained from Genzyme.

NO_2^- assay

The determination of NO_2^- was performed in culture supernatants of AM stimulated or not with IFN-γ and challenged or not with *C. albicans* or encapsulated or acapsular *C. neoformans* as previously described [20]. Briefly, 50 μl of cell-free supernatants were incubated with 50 μl of Griess reagent (1% sulphanilamide/0·5% naphthylendiamine dihydrochloride/2·5% H_3PO_4) at room temperature for 10 min and the absorbance at 550 nm was determined in a Sorin Biomedica microplate reader. The concentration of NO_2^- was determined from a standard curve which was linear between 10 and 200 μM sodium nitrite. All samples were performed in duplicate.

Preparation of granule extracts

Preparation of granule extracts was performed as previously described [16]. Granule extracts were isolated from AM from three selected BAL in which the cells harvested were more than 2×10^7. Briefly, AM were activated with IFN-γ for 18 h and collected in PBS pH 7·4 by scraping with a rubber policeman. After washing with PBS, AM were suspended at 1×10^7 cells/1·0 ml of 0·34 M sucrose and disrupted with a Teflon pestle homogenizer. The homogenate was centrifuged at 900 *g* for 10 min at 4°C, and the supernatant, which was rich in cytoplasmic granules, was centrifuged at 27 000 *g* for 20 min at 4°C. The granule pellet was stored at −20°C. For extraction, granules were suspended in 0·3 ml of 0·01 M citric acid pH 2·7 and stirred for 2 h in an ice bath. The protein contents of the extracts were determined by the method described elsewhere [16] with BSA (Sigma) as the standard.

Fungicidal activity of granule extracts

Candida albicans or *C. neoformans* (6995 or 7698) cells were washed twice with 0·01 M PBS pH 7·4 and suspended in PBS. Assay mixtures consisted of 5×10^4 yeast cells in phosphate buffer and granule extracts at a concentration of 5 μg protein/ml (total volume 0·5 ml). For characterization of the fungicidal extracts, trypsin (500 μg/ml; Sigma) or EDTA (0·01%; Sigma) was added to the assay mixtures. The 0·01 M citric acid solution was added to each control. After incubation for 1 h at 37°C, the samples were serially diluted and inoculated onto Sabouraud agar plates. The plates were incubated at 37°C for 24 h for *Candida* and at 28°C for 72 h for *C. neoformans*, and the resulting colonies were counted. To determine the fungicidal activity of granule extracts at pH 3, 0·01 M citrate buffer pH 3·0 was used for the control.

Statistical analysis

Differences between the mean values were analysed by Student's *t*-test.

Table 1. Phagocytic and killing activity of human alveolar macrophages (AM), peripheral blood monocytes (PBM) and polymorphonuclear (PMN) cells *versus Candida albicans* or *Cryptococcus neoformans* encapsulated (6995) or acapsular (7698) strains

	Per cent phagocytic cells			Per cent killing activity		
		CN			CN	
	CA	6995	7698	CA	6995	7698
AM	68·2 ± 5·6	55·6 ± 6·9	61·9 ± 7·3	45·8 ± 5·3	18·9 ± 4·1	46·4 ± 4·0
PBM	70·5 ± 6·8	62·3 ± 5·8	70·2 ± 5·9	55·3 ± 7·4	38·3 ± 5·1*	60·5 ± 3·2*
PMN	72·3 ± 8·4	65·3 ± 6·5	64·5 ± 6·3	69·2 ± 5·8*	61·9 ± 5·3*	68·5 ± 4·3*

Phagocytic activity was evaluated as a per cent of phagocytosis at an effector-to-target ratio of 1:10 in 2 h of incubation at 37°C in 5% CO_2. The results represent the mean of four separate experiments from different donors.

*$P < 0·01$ (PBM and PMN *versus* AM).

CA, *C. albicans*; CN, *C. neoformans*.

Table 2. Time course of proliferative response of T lymphocytes to *Cryptococcus*-laden (6995 or 7698) alveolar macrophages (AM) or peripheral blood monocytes (PBM)

Cells	*Cryptococcus* addition	Days of culture (mean of ct/min ± s.d.)		
		+1	+3	+7
AM + T(E^+)	–	205 ± 75	305 ± 99	205 ± 95
AM + T(E^+)	6995	593 ± 82	8421 ± 529	9884 ± 502
AM + T(E^+)	7698	891 ± 80	15834 ± 893*	23383 ± 2189*
PBM + T(E^+)	–	691 ± 58	594 ± 102	485 ± 91
PBM + T(E^+)	6995	840 ± 391	16852 ± 2530	20832 ± 3502
PBM + T(E^+)	7698	1984 ± 256	23840 ± 3680*	36860 ± 4520*

AM or PBM (2×10^4), in the presence or absence of (2×10^5) heat-inactivated *C. neoformans* encapsulated (6995) or acapsular (7698) strains, were co-cultured with autologous T(E^+) cells (1×10^5). Proliferation was measured by ^{3}H-thymidine incorporation at various days (+1, +3, +7) of culture. Ct/min represent the mean of four experiments from four different subjects.

* $P < 0.01$ (7698 culture-treated *versus* 6995-treated).

RESULTS

In order to evaluate the role of the capsule on destructive activity of natural effectors, we compared fungicidal activity of AM, PBM and PMN *versus* the encapsulated (6995) or acapsular (7698) strain of *C. neoformans* or *C. albicans*. Table 1 shows that phagocytic activity was similar in *C. albicans* and *C. neoformans* encapsulated (6995) or acapsular (7698) strains, while killing activity was appreciable only when *C. albicans* and acapsular *C. neoformans* 7698 were used. Moreover, antifungal activity of AM was significantly impaired with respect to other natural effectors such as PBM or PMN cells. In a previous paper, we demonstrated that unstimulated human AM play an important role as APC in *C. neoformans* infection, inducing proliferation of α/β TCR^+ T cells [17]. To evaluate the role of the capsule in the development of immune response mediated by cryptococcus-laden AM, we performed experiments using encapsulated or acapsular *C. neoformans*. Table 2 shows that AM after phagocytosis of *C. neoformans* induce a proliferative response of autologous T lymphocytes significantly higher when the acapsular strain is employed; a similar phenomenon was observed when PBM were used as APC. To verify the phenotype of proliferating cells, T lymphocytes were harvested after 7 days of culture and analysed by FACScan using anti-human CD3, CD4 and CD8 FITC conjugates. The results obtained show that (Fig. 1) $CD4^+$ was the T cell population mainly involved in response to *C. neoformans*, even if a small percentage of CD8 (10–15%) was present. To characterize the $CD4^+$ T lymphocyte subpopulation that played a major role in this response, we determined cytokine levels in supernatant co-culture of *Cryptococcus*-laden AM plus T lymphocytes. The results reported in Table 3 show that high levels of IL-2 and IFN-γ were found, while IL-4 was undetectable. High levels of IL-2 and IFN-γ were found in the early phase of infection (after 1 day of culture) and remained high for up to 7 days. It is worth noting that the amount of cytokine production by T lymphocytes in the presence of the acapsular strain was significantly higher than that secreted in the presence of the encapsulated yeast. Since IFN-γ is a potent inducer of microbicidal activity in macrophages, we tested the ability of this cytokine to modulate anti-cryptococcal activity. The results reported in Table 4 show that the addition of IFN-γ *in vitro* to AM was able to induce killing activity *versus* encapsulated *C. neoformans* and augment microbicidal activity *versus C. albicans* and acapsular *C. neoformans*. Very small amounts of NO_2^- were recovered in supernatants of AM infected with *C. albicans* or *C. neoformans* 6995 or 7698 strains, and did not increase when the cells were stimulated with IFN-γ. By contrast, a good correlation between fungicidal activity of AM and microbicidal properties of their cytoplasmic granule extracts was found

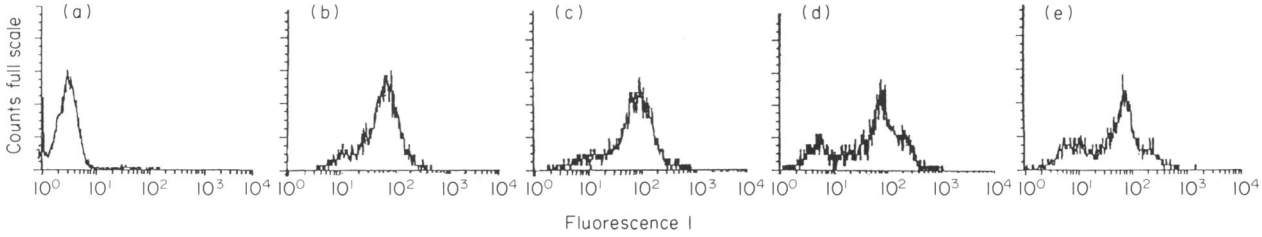

Fig. 1. Cytofluorimetric analysis of proliferating T cells in response to *Cryptococcus* (6995 or 7698)-laden alveolar macrophages (AM). (a) Proliferating T cells stained with FITC anti-IgG. (b) Proliferating T cells to *Cryptococcus* 6995-laden AM stained with FITC anti-CD3 antibodies. (c) Proliferating T cells to *Cryptococcus* 7689-laden AM stained with FITC anti-CD3 antibodies. (d) Proliferating T cells to *Cryptococcus* 6995-laden AM stained with FITC anti-CD4 antibodies. (e) Proliferating T cells to *Cryptococcus* 7689-laden AM stained with FITC anti-CD4 antibodies.

Table 3. IL-2, IFN-γ and IL-4 production by T lymphocytes co-cultured with *Cryptococcus*-laden alveolar macrophages (AM)

Days of culture	IL-2, pg/ml			IFN-γ, pg/ml			IL-4, ng/ml		
	NS	6995	7698	NS	6995	7698	NS	6995	7698
1	0·0	70·3 ± 5·8	90·3 ± 4·0	1·6 ± 0·6	39·2 ± 2·3	55·3 ± 3·0	0·00	0·07	0·04
3	2·0	53·0 ± 4·9	72·5 ± 6·8	0·9 ± 0·1	65·1 ± 8·4	94·2 ± 6·9	0·01	0·06	0·04
7	0·1	50·1 ± 6·8	77·3 ± 7·0	1·8 ± 0·8	66·0 ± 6·3	98·3 ± 7·0	0·07	0·14	0·05

IL-2, IFN-γ and IL-4 were measured in supernatants of cocultures of *Cryptococcus*-laden AM (encapsulated 6995 or acapsular 7698) plus T lymphocytes. The results represent the mean of four separate experiments from different donors.

NS, Unstimulated cells.

after IFN-γ stimulation. Trypsin or EDTA treatment abrogated the above described effect. Hence, IFN-γ-mediated activation in AM is related to an enhancement of microbicidal properties of cationic-activated proteases.

DISCUSSION

This study provides evidence that unstimulated AM are fungicidal *versus* the acapsular strain of *C. neoformans*, and killing is minimal when the encapsulated yeast is used. AM seem to be less efficient in killing *C. neoformans* compared with other natural effectors such as PBM or PMN. After phagocytosis AM induce a proliferative response of autologous $CD4^+$ T lymphocytes that secrete IL-2 and IFN-γ, but not IL-4. The amount of cytokine secretion is significantly enhanced when the acapsular strain is used compared with the encapsulated yeast. Secretion of IFN-γ is crucial to induce AM fungicidal activity *versus* encapsulated *C. neoformans*, and killing is independent of nitric oxide (NO_2^-) production, but mediated by an enhancement of destructive activity of cytoplasmic granule extracts.

Cryptococcus neoformans enters the body by the inhalatory route and can disseminate from the lung to the central nervous system [21]. The infection in immunocompetent hosts is rare, but in immunodepressive conditions such as AIDS, it causes meningoencephalitis [22]. AM represent the first line of defence against *C. neoformans* and seem to be less efficient in killing this fungus than PBM and PMN. These latter cells could be responsible for the control of yeast cells that escape from the lung and enter the circulation, as suggested by Miller & Mitchell [23]. Despite their poor function as effector cells, unstimulated AM could very efficiently phagocytize the fungus, and their function as APC may play a pivotal role in defence against *C. neoformans*. In fact, these cells trigger lymphoproliferation, which is particularly evident when the acapsular strain is employed. Our results are in agreement with those reported by Collins & Bancroft on the impairment of specific T cell response that is ascribed to encapsulation of *C. neoformans* [14]. Mody & Syme also describe an immunosuppressive effect on lymphoproliferation attributable to capsular material [15]. We demonstrate that this phenomenon is also present when AM are used as APC. In our opinion, AM

Table 4. Correlation between killing activity of alveolar macrophages (AM) and microbicidal activity of their granule extracts

Cells	*In vitro* stimulation	Target cells	Killing* activity of AM	Killing activity† of granule extract	NO_2^-‡ production (10^7 cells)
AM	–	CA	45·3 ± 5·8	53·3 ± 3·9	21·3 ± 3·9
AM	IFN-γ	CA	61·9 ± 5·2§	66·2 ± 4·0§	20·4 ± 2·5
AM	–	6995	12·0 ± 3·8	24·2 ± 3·2	28·8 ± 5·0
AM	IFN-γ	6995	28·4 ± 4·0§	37·1 ± 2·3§	21·6 ± 4·2
AM	–	7698	39·7 ± 3·5	34·3 ± 4·2	22·8 ± 4·3
AM	IFN-γ	7698	51·3 ± 2·9§	47·0 ± 2·1§	32·7 ± 6·3

AM were treated for 18 h with IFN-γ (500 U/ml) and then treated with *Candida albicans* or *Cryptococcus neoformans* 6995 or 7698 for 6 h.

* Killing activity of AM was evaluated in unstimulated or IFN-γ-stimulated cells as a percentage of colony-forming unit (CFU) inhibition at an effector-to-target ratio of 10:1 cells.

† Killing activity of granule extracts was evaluated at 5 μg/ml of protein concentration in 0·01 M phosphate buffer pH 7·4 as described in Materials and Methods.

‡ Determination of NO_2^- was performed in supernatants of AM or AM + IFN-γ with or without target cells. The $NONO_2^-$ production without target cells from 10^7 AM unstimulated cells was 19·2 ± 4·8 and from 10^7 AM stimulated with IFN-γ was 21·3 ± 5·0.

§ $P < 0·01$ (IFN-γ-treated cells *versus* untreated).

process and present cryptococcal protein antigens of the acapsular strain more efficiently than those of the encapsulated yeast.

In a previous paper, we demonstrated that *Cryptococcus*-laden AM are able to induce a proliferative response of α/β T lymphocytes [17]. In the present study, we demonstrate that these cells are mainly $CD4^+$ (80–85%) with a small percentage of $CD8^+$ (10–15%). This pattern of response is similar in both strains of *C. neoformans*.

During lymphoproliferation in co-culture of *Cryptococcus*-laden AM plus T lymphocytes, higher levels of IL-2 and IFN-γ, but not IL-4, were detected, suggesting that the $CD4^+$ subset, mainly involved in this response, conforms to a Th1 population on the basis of the profile of cytokine production [24]. Moreover, IL-2 levels are significantly higher when the acapsular strain is used compared with the encapsulated yeast. This phenomenon could, in part, explain the augmented lymphoproliferation observed with the acapsular strain. Our results are in line with those recently reported by Murphy in a murine model, in which the induction of a cell-mediated immune response against *C. neoformans* is associated with IL-2 and IFN-γ production by splenocytes [25].

Endogenous IL-2 at lung level could be important since this cytokine induces anti-cryptococcal activity through conjugate formation in human T cells, and the presence of T lymphocytes is required in controlling pulmonary infection [26]. Since AM are only poorly able to kill encapsulated *C. neoformans* [17], it is possible that this fungus can replicate in unstimulated macrophages [27–29]; lymphocyte proliferation triggered by *Cryptococcus*-laden AM is crucial to induce IL-2 and IFN-γ which, in turn, could induce fungicidal activity in T cells and AM, respectively. It is possible that cytokine production by the Th1 subset in the early stages of infection down-regulates the Th2 response that develops humoral rather than cellular immunity. This could lead to the inhibition of macrophage function and progression of infection. Th2 activation could be related to AIDS, in that the susceptibility to *C. neoformans* infection might be due to a loss of the ability of Th1 cell development and IL-2 production to recall antigens, resulting in an increase of IL-4 production which acts as a negative feed back by inhibiting Th1 cells. The fungicidal mechanisms induced in AM by IFN-γ are correlated with the enhancement of killing activity of cytoplasmic cationic proteases, but this phenomenon does not exclude an extracellular killing mediated by secreted proteins, as suggested by Flesch *et al.* in a murine model [30]. By contrast, NO_2^- production is not modulated, suggesting that NO_2^- does not play a role in the activation of human AM by IFN-γ, even if a mechanism implicating NO_2^- has been described in a murine model in killing of *C. neoformans* [31,32]. The pathobiological significance of results obtained *in vitro* should be investigated in an *in vivo* system. It seems that local immunity at lung level is crucial for the outcome of *C. neoformans* infection [33–35].

Taken together, these results show that *Cryptococcus*-laden AM induce autologous $CD4^+$ T cell proliferation with induction of IL-2 and IFN-γ; the level of cytokine production and lymphoproliferation is dependent upon the encapsulation of *C. neoformans*. The amount of IL-2 and IFN-γ could be important in the activation of destructive activity of AM and T cells which are present in the lung in *C. neoformans* infection. The possibility of up-regulation of these cytokines could be taken into account in the therapeutic approach to controlling infection in the immunocompromised host.

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