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The *Candida albicans* phospholipomannan induces *in vitro* production of tumour necrosis factor-α from human and murine macrophages

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SUMMARY

We have previously identified a *Candida albicans* 14 000–18 000 MW antigen reacting with anti-β-1,2-linked oligomannosides antibodies as being a phospholipomannan (PLM). Because of the structural similarities between the *C. albicans* PLM and lipophosphoglycans from various microbial pathogens known to be potent tumour necrosis factor-α (TNF-α) inducers, we investigated the PLM ability to induce TNF-α. Incubation of human monocytic cells THP-1 with PLM led to dose-dependent production of TNF-α that was significantly increased by prestimulation of the cells with interferon-γ (IFN-γ). Production of TNF-α by macrophages under PLM stimulation was confirmed by using macrophages elicited from the mouse peritoneal cavity. In all investigated conditions, PLM-induced TNF-α production differed significantly in both kinetics and dose dependence from lipopolysaccharide (LPS) induction used as control. It appears, therefore, that the *C. albicans* PLM shares functional homologies with microbial lipophosphoglycans identified as pathogenicity factors, although prestimulation of the target cells was required for the PLM-derived opportunistic pathogen to trigger the cytokine network.

INTRODUCTION

Over the past few years, there has been a growing body of scientific evidence to suggest that the opportunistic yeast *Candida albicans* might share the ability to induce cytokines involved in pathological processes with unicellular pathogens. Such an effect has been well documented *in vivo* for tumour necrosis factor-α (TNF-α) production following experimental infection.[1,2] Several other experiments have shown that this phenomenon could be reproduced *in vitro*, by using cells of the monocytic lineage as targets for infection.[3–6] Induction of TNF-α production by *C. albicans* is worth exploring because of the microbicidal activities[7] but also in relation to the possible implication of this cytokine in immunopathological processes observed during the time–course of human, or other animal, *Candida* septicaemia.[8,9] However, few data are available concerning the identification of molecules responsible for these effects. At the moment, the most precise information originates from Vecchiarelli *et al.*[5] who have demonstrated that cell wall-extracted mannoproteins induced TNF-α production by mouse splenic macrophages.

Following an analysis of the distribution of oligomannosidic epitopes over *C. albicans* molecules,[10,11] we have recently identified a 14 000–18 000 MW antigen, unreactive to concanavalin A (Con A), whose recognition through metaperiodate-sensitive epitopes was restricted to monoclonal or polyclonal antibodies having the property of reacting with homopolymers of β-1,2-linked mannopyranosyl residues.[11,12] Subsequent analyses have led to the identification of the 14 000–18 000 MW antigen as a phospholipomannan (PLM), according to its resistance to protease digestion, extraction by chloroform/methanol/water (C/M/W) and presence of phosphate, mannose and palmitic acid, as demonstrated by metabolic labelling.[13] Throughout this characterization, the *C. albicans* PLM revealed structural homologies with molecules distributed over a wide variety of microbial pathogens, such as the *Leishmania donovani* lipophosphoglycan (LPG),[14] the *Mycobacterium tuberculosis* lipoarabinomannan (LAM)[15] or the so-called 'toxin' of *Plasmodium falciparum*.[16] All these molecules, which share glycan moieties associated with lipid and phosphorus, have been demonstrated to be potent TNF-α inducers.[14–16]

We therefore investigated in the present study, the ability of the *C. albicans* PLM to induce TNF-α by using *in vitro* models of human and mouse cells of the monocytic lineages. In these models we compared TNF-α induction resulting from PLM stimulation with TNF-α production under bacterial lipopolysaccharide (LPS) stimulation.[17]

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Abbreviations: C/M/W, chloroform/methanol/water; FCS, fetal calf serum; IFN-γ, interferon-γ; IL, interleukin; LAM, lipoarabinomannan; LPG, lipophosphoglycan; LPS, lipopolysaccharide; PLM, phospholipomannan; TNF-α, tumour necrosis factor-α.

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MATERIALS AND METHODS

Reagents

LPS from *Escherichia coli* (serotype 0111:B4) and Polymyxin B sulphate (6000 U/mg) were obtained from Sigma Chemical (St Louis, MO). Recombinant human TNF-α (specific activity 2×10^7 U/mg) was obtained from Genzyme Corp. (Cambridge, MA). Recombinant human interferon-γ (IFN-γ; specific activity 2×10^7 U/mg) was from Roussel-Uclaff (Romainville, France). Actinomycin D was obtained from Calbiochem-Behring Corp. (La Jolla, CA).

Candida albicans *phospholipomannan*

The *C. albicans* PLM was purified from the VW32 strain of *C. albicans*, as described elsewhere.[13] Briefly, cells were grown in a 50-l bioreactor.[10] Ten grams of yeast cells (dry weight) was suspended in 50 ml phosphate-buffered saline (PBS) and broken by two consecutive runs in a French pressure cell at 20 000 psi. Cell extracts containing cell wall and cell membranes were then dialysed overnight and lyophilized. These cell extracts were treated at room temperature three times with 100 ml chloroform/methanol (2/1) (total time: 30 hr) to remove lipids and phospholipids. They were then treated under the same conditions with C/M/W (10/10/1) to remove less polar glycolipids, and finally with C/M/W (10/10/3) to extract more polar glycolipids, which were pooled, dried and treated with proteinase K 0·5 mg/ml in 1 mM EDTA, 10 mM Tris–HCl buffer, pH 8, for 1 hr at 60°. The dried samples were then extracted again with C/M/W (10/10/3) to remove proteinase K. After desiccation in a speedvac, the PLM stock solution contained 2·5 mg/ml sugars, as estimated by the phenol sulphuric method. Appropriate PLM concentrations, which corresponded to 1 : 500 to 1 : 50 000 dilutions of the PLM stock solution in culture medium, were prepared after sonication in a water bath.

All reagents used in the studies were tested in two different Limulus assays for detection of endotoxin. In these tests, optimal concentrations of PLM preparations used in the study contained less than 0·2 EU/ml corresponding to 10 000-fold less than the endotoxin activity measured in the LPS preparations used as references.

Cell lines and animal-derived cells

The monocytic cell line THP-1[18] was cultured at $0{\cdot}5 \times 10^6$ cells/ml at 37° in 5% CO_2, in RPMI-1640 (Flow Laboratories, Rockville, MD) supplemented with 10% fetal calf serum (FCS), 1% glutamine and 1% antibiotic mixture. The TNF-sensitive L929 mouse fibroblasts were maintained in DMEM (Gibco Laboratories, Grand Island, NY) containing 5% FCS and antibiotic mixture.

Six- to 8-week-old female BALB/c mice were obtained from IFFA-CREDO (L'Arbresle, France). Peritoneal exudate cells (PEC) were elicited by injecting two to three mice intraperitoneally with 1 ml of sterile 10% proteose peptone broth (Difco Laboratories, Detroit, MI) 72 hr before assay. Mice were killed by cervical dislocation and the PEC were recovered by rinsing of the peritoneal cavity with 5 ml RPMI. Cells were washed twice in RPMI containing 5% FCS and adjusted to a concentration of 5×10^6 cells/ml. One hundred-microlitre quantities of this cell suspension were then distributed in 96-well plates and incubated for 3 hr at 37° in 5% CO_2 to permit adherence of the cells. Medium and non-adherent cells were then removed by aspiration and vigorously washed twice in warm culture medium.

Production of TNF-α

Mouse macrophage monolayers were incubated in 96-well culture plates with 150 μl of RPMI 5% FCS containing different dilutions of LPS, or of *C. albicans*-derived antigens, for 18 hr at 37°. THP-1 cells ($0{\cdot}5 \times 10^6$ cells/ml) were incubated in 24-well plates in the presence of 500 μl culture medium containing different dilutions of either LPS or *C. albicans*-derived PLM. In some experiments, THP-1 cells were activated for 3 hr at 37° with IFN-γ (100, 500 and 1000 U/ml) prior to the addition of antigens. Control experiments involved incubation with antigens in the presence of 20 μg/ml of Polymyxin B. At different time periods, supernatants from conditioned cultures were collected and frozen at −70° for subsequent analysis. After treatment, the cell viability was similar to that of control cells (i.e. > 95%, as determined by trypan blue dye exclusion).

Measurement of cytokine production

TNF production in cell-free supernatants of conditioned culture medium was determined following a lytic assay of the TNF-sensitive mouse fibroblasts, L929, as already described.[19] Briefly, 2×10^4 L929 cells in 100 μl of culture medium were allowed to adhere at 37° overnight in 96-well culture plates. Growth medium was then removed and 50 μl of fresh culture medium was added to the cells. Fifty microlitres of twofold serially diluted conditioned supernatants was then added. Finally, 50 μl of culture medium containing 4 μg/ml actinomycin D was added to each well. Cellular death was determined after 24 hr incubation at 37°; after washing in PBS, residual living adherent cells were fixed for 20 min with glutaraldehyde (3% in PBS) and stained for 30 min with methylene blue (2% in water). Stained cells were then washed and eluted in 100 μl of 0·1 N HCl. Absorbency at 620 nm was then measured using a microplate reader. In each plate, negative control (0% lysis), consisting of cells incubated with medium alone, and positive control (100% lysis), obtained by incubating the cells in the presence of 2% triton, were incorporated. The TNF-α concentration of tested cultures was evaluated on the basis of cellular death obtained in the same conditions in the presence of serially diluted recombinant TNF-α (from 400 to 3 pg/ml). By using this method, the lower limit of TNF-α detectability was 5–10 pg/ml (0·5–1 pg/well), and the dose of TNF-α leading to 50% cell mortality (defined as 1 U) corresponded to 25 pg/ml (2·5 pg/well). The sensitivity and the specificity of the assay, and the integrity of the TNF-α produced by the cells, were verified by comparison of the results obtained with the same supernatants analysed using an ELISA whose sensitivity was 10 pg/ml TNF-α (Eurogenetics, Orléans, France) and by neutralization of cell cytotoxicity obtained after addition to the supernatants of a 1:200 dilution of polyclonal rabbit anti-TNF-α (Genzyme Corp.).

RESULTS

TNF-α production by THP-1 cells following incubation with LPS or PLM

To evaluate the ability of the THP-1 cell line to produce TNF-α, we first examined the effect of LPS stimulation on secretion of

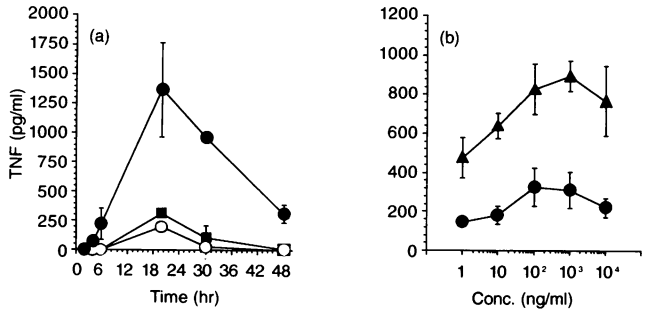

Figure 1. Effect of PLM and LPS incubation on TNF-α production by THP-1 cells. (a) Kinetics of TNF-α production by THP-1 cells incubated for different time periods in the absence (open symbols) or presence (closed symbols) of 1 μg/ml LPS (circles) or 10 μg/ml PLM (squares). (b) THP-1 cells were cultured with the indicated concentrations of LPS (triangles) or PLM (circles) and TNF-α release was measured after 18 hr incubation. Results expressed as the mean ± SD of 1 determination are representative of at least four independent experiments.

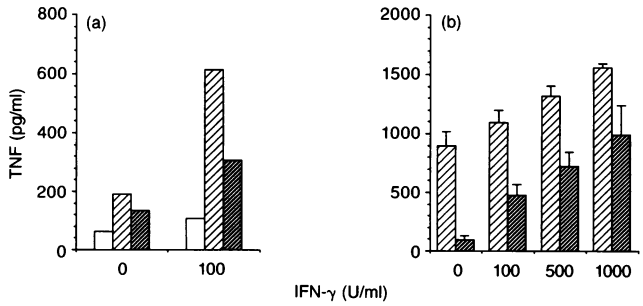

Figure 2. Effect of IFN-γ activation of THP-1 cells on TNF-α production. (a) THP-1 cells were incubated for 3 hr in the presence or absence of 100 U/ml of IFN-γ. Cells were then incubated either in medium alone (open), in the presence of 0·5 μg/ml LPS (light shading) or 0·5 μg/ml PLM (dark shading). TNF-α concentrations were determined after 18 hr. (b) THP-1 cells were incubated with 0·5 μg/ml LPS (light shading) or 0·5 μg/ml PLM (dark shading) after prestimulation with different concentrations of IFN-γ. The results shown are representative of three independent experiments.

this cytokine. In the experimental conditions used (Fig. 1a), THP-1 cells *per se* produced low amounts of TNF-α, reaching a maximum level after 20 hr incubation. In contrast, stimulation of the cells with 1 μg/ml LPS led to the detection of significant levels of TNF-α as early as 4–6 hr after stimulation, which increased to a maximum over 18–20 hr before slowly decreasing. At the time of maximal production (Fig. 1b), the amount of secreted TNF-α was shown to be dependent on the amount of LPS, maximum secretion being observed with 1 μg/ml LPS. These results therefore demonstrated that, as with other monocytic cells, THP-1 cells were able to secrete TNF-α in response to a stimulus such as LPS. When the same model was used to investigate the effect of different PLM concentrations on the TNF-α secretion after incubation for different periods of time, only limited amounts of TNF-α were detectable in comparison to LPS stimulation (Fig. 1a). Following 18 hr of incubation, it was nevertheless possible to demonstrate a PLM dose-dependent secretion of TNF-α that reached a maximum when cells were incubated with a concentration of 100 ng/ml (Fig. 1b). These results show that PLM was able to induce THP-1 cells to secrete TNF-α, although being a far less potent stimulator than LPS. LPS doses as low as 1 ng/ml induced a level of TNF-α production that was not reached even with up to 10^4-fold higher PLM concentrations. These data confirmed the absence of LPS contamination of the PLM preparation, as initially established with endotoxin detection assays, but they also raised the question about the possible existence of a PLM TNF-α induction pathway differing from that used by LPS. This difference was confirmed when cells were incubated with antigens in the presence of Polymyxin B to inhibit LPS-dependent TNF-α induction of the cells. In this case, whereas cytokine production after LPS stimulation could be inhibited (39·2 ± 6·7%), no significant difference was observed when cells were incubated with PLM in the presence or absence of Polymyxin B (5·8 ± 6·7%).

Enhancement of PLM-dependent TNF-α production following activation of THP-1 cells by IFN-γ

In an attempt to explore further the influence of cell activation status on TNF-α production induced by PLM, THP-1 cells were first activated by preincubation in the presence of IFN-γ. Preliminary experiments showed that a 3-hr preincubation of the cells in the presence of 100 U/ml IFN-γ (Fig. 2a) resulted in a constitutive production of TNF-α after a 18-hr incubation, even in the absence of any stimulus. However, addition of PLM (0·5 μg/ml) during this second incubation step resulted in a marked increase of TNF-α production when compared to the control and to LPS-stimulated cells (Fig. 2a). Subsequent experiments (Fig. 2b) demonstrated that this TNF-α production in the presence of both LPS and PLM was dependent on the dose of IFN-γ used to preactivate the cells.

Induction of TNF-α production following incubation of mouse macrophages with PLM

Inasmuch as cell activation status appeared to be important for PLM stimulation, the effect of PLM on the TNF-α production was finally examined on monocyte-derived macrophages elicited in the peritoneal cavities of mice and purified by adherence. In contrast with THP-1 cells, cells incubated with medium alone secreted TNF-α: high amounts of this cytokine were detectable after as little as 2 hr of incubation, but rapidly decreased to low or undetectable levels after 8 hr of culture (Fig. 3a). By comparison, incubation with LPS led to the secretion of a higher level of TNF-α, even after 2 hr incubation, and maximal production was obtained after 8 hr before decreasing after 18 hr of culture. When PLM was used to stimulate the cells, significant TNF-α production was obtained only after 18 hr of incubation (Fig. 3a). At this time, TNF-α production was significantly different from that obtained with control cells and was dependent on the dose of PLM, cytokine secretion being maximal with 0·5 μg/ml PLM (Fig. 3b). Thus, as with THP-1 cells, PLM induced TNF-α secretion by mouse macrophages differing from that obtained with LPS, both at the level of the time period necessary to gain optimal stimulation and at the level of the amount of cytokine produced by the cells.

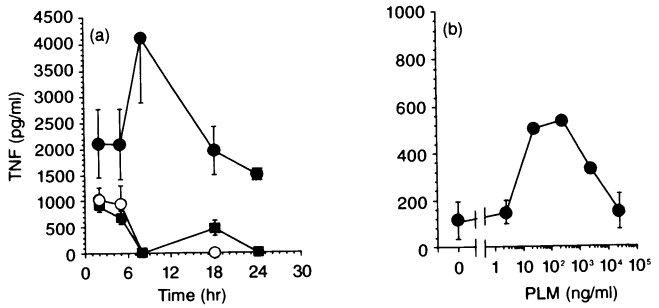

Figure 3. Effect of PLM incubation on TNF-α secretion by mouse macrophages. (a) Kinetics of TNF-α production after incubation of purified monocytic cells without (open symbols) or with 0·5 μg/ml LPS (closed circles) or PLM (closed squares). (b) TNF-α production by monocytic cells after stimulation for 18 hr in the presence of different concentrations of PLM. Data of triplicate determinations of one experiment are representative of two separate experiments.

DISCUSSION

Several studies, performed either *in vivo* or *in vitro*, have demonstrated that *C. albicans* was able to initiate a cytokine network.[1–3,20] Among the cytokines induced by *C. albicans*, the largest number of reports concern TNF-α, a cytokine which, together with interleukin-1 (IL-1), represents the first molecule produced by cells of the monocytic lineage involved in the enhancement of the microbicidal activities.[9] A role for TNF-α in relation to phagocytic clearance of *C. albicans* has been suggested in experimental models of candidiasis.[2,21] However, TNF-α also acts as a mediator of septic shock, either directly or by initiating an immunological cascade that leads to the appearance in the serum of acute phase proteins and other cytokines.[9] The possible implication of TNF-α production in mortality and morbidity of *C. albicans*-infected animals has been suggested in both mouse and rat experimental models;[1,2,20] and a relationship between infection, cytokine production and synthesis of acute phase proteins has been established.[1,22] Concerning human infections, TNF-α induction by *C. albicans* may be important to consider in clinical circumstances relating to candidaemia and/or *Candida* septicaemia. These clinical entities, now precisely defined,[23] are frequently observed in hospital environments. They present with clinical features so closely related to septic syndromes due to Gram-negative bacteria that the differential diagnosis is difficult to establish in the absence of mycological evidence of *Candida* in the blood stream.

However, limited information is available concerning the *C. albicans* components responsible for TNF-α induction. At the moment, morphological transition from yeast to hyphae has been demonstrated to increase *C. albicans*' ability to induce TNF-α.[24] Viable yeast cells have been shown to be more potent inducers than heat-killed cells *in vivo*,[2] although converse results have been obtained *in vitro*.[4] At the molecular level, the most precise information concerns two yeast cell wall mannoproteins, separated by ion-exchange chromatography, which both mimicked the effect of yeast-killed cells on TNF-α induction by splenic macrophages.[5]

We have recently described a *C. albicans* PLM which appears in SDS–PAGE as a quantitatively important 14 000–18 000 MW component, and may be purified through methods of glycolipid extraction and subsequent treatment by broad spectrum proteases.[13] The PLM polysaccharidic moiety contains Con A-unreactive mannose residues recognized by monoclonal or polyclonal antibodies reacting with homopolymers of β-1,2-linked mannose.[11–13] The PLM is expressed by both yeast and mycelial forms of *C. albicans*[13] and, among the genus *Candida*, its presence is restricted to the more pathogenic species *C. albicans* and *C. tropicalis* (C. Cantelli, P. A. Trinel, A. Bernigaud & D. Poulain, manuscript submitted for publication). Through its gross chemical composition,[13] the *C. albicans* PLM shows homologies with glycolipids recently identified from a wide variety of pathogenic micro-organisms. Among these molecules, which all consist of glycan moieties associated with phosphorus and lipids, the most characterized are LAM from *Mycobacterium tuberculosis*,[15] LPG from *Leishmania donovani*,[14] and the so called 'toxin' from *Plasmodium falciparum*.[16] All these structurally related molecules share the biological property of being potent cytokine inducers, and the data support the idea that they are involved, through this property, in the development of pathological processes.[14–16]

In the investigation of *C. albicans* PLM ability to induce TNF-α, we used *in vitro* models involving cells of the monocytic lineage in comparison with the bacterial LPS, the so-far better characterized TNF-α inducer.[17] High amounts of TNF-α were obtained after stimulation by LPS of the human monocytic cell line THP-1.[18] However, the time–course of production and dose dependence were different from those obtained with other monocytic cells described elsewhere[6] and in our own experiments with mouse macrophages. This difference in kinetics was consistent with other experiments using this cell line[25] in which maximum stimulation with LPS was obtained after 24 hr. In comparison, we found that *C. albicans* PLM induced a dose-dependent production of TNF-α. Although PLM appeared to be a less potent TNF-α inducer than LPS, it is of note that both the kinetics and amounts of cytokine produced were similar to those obtained by others[25] when THP-1 cells were stimulated with the well-characterized LAM TNF-α inducer from *M. tuberculosis*.

In view of cell maturation dependence for TNF-α secretion,[17] we then addressed the question of whether activation of THP-1 cells could lead to an enhanced susceptibility of cells to PLM stimulation. For this purpose, we used IFN-γ, a lymphokine which has been described to bind at the membrane of THP-1 cells and to induce their maturation.[26] As previously described,[27] we have found that pretreatment of THP-1 cells with IFN-γ led to increased TNF-α production, even in the absence of any other stimulation. However, LPS stimulation resulted in higher amounts of TNF-α released in the culture medium. This increased production of cytokine was also observed when cells were secondarily incubated with PLM, thus demonstrating an up-regulation by IFN-γ of THP-1 cells, PLM-induced TNF-α production. However, it is impossible at present to define the events involved in this enhancement, since it has been shown that IFN-γ may act either directly at the mRNA level,[28] or on the increased expression of membrane receptors involved in signal transduction.[29]

When mouse macrophages were used, the PLM ability to induce TNF-α on matured cells was confirmed, as well as the existence of strong differences between the two inducers under study. With these cells, TNF-α was produced as early as 5–8 hr under LPS stimulation, while significant levels of cytokine were

obtained only after a 18-hr incubation when PLM was used. One of the reasons for this delayed production could be the relative concentrations of the two components but, as observed with the THP-1 cells, the highest dose of PLM did not produce higher amounts of TNF-α. These data are consistent with results reported by Blasi *et al.*[6] who use the mouse monocytic cell line ANA-1 incubated with live *C. albicans* yeast cells: maximal yeast concentrations also led to a lower stimulation of TNF-α production. Differences observed in TNF-α production under PLM and LPS stimulation, which concern both kinetics and dose-dependence, thus suggest different pathways for the two stimuli. Such a difference has already been observed at the mRNA level for TNF-α production by cells stimulated either with LPS or with *L. donovani* LPG.[14] Investigations are in progress to explore the possible existence of a pathway or receptor specific for *C. albicans* PLM TNF-α induction in monocytic cells. Interestingly a receptor for *C. albicans*-derived β-1,2-linked oligomannosides has been described recently at the membrane of mouse splenic macrophages.[30] Since we have shown that the PLM glycan moiety presents with this special type of linked mannopyranosyl residues,[13] the possible identity between this receptor and the one triggering the monocytic cells for TNF-α production will be investigated jointly.

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