Lymphoproliferative and cytotoxic responses of human peripheral blood mononuclear cells to mannoprotein constituents of *Candida albicans*

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Two major proteoglycan constituents (designated F1 and F2) of the cell wall of *Candida albicans* were separated by ion-exchange chromatography from a crude carbohydrate-rich extract (GMP), and investigated for their chemical and molecular composition, antigenicity and immunomodulatory properties in cultures of human peripheral blood mononuclear cells (PBMC). Both fractions consisted predominantly of Periodic acid-Schiff (PAS) and concanavalin A (Con A)-reactive material consisting of >90% mannose, 3–5% protein and small amounts of phosphorus; each was recognized by an anti-*Candida* rabbit serum as well as by a monoclonal antibody (mAb AF1) directed against an oligosaccharide epitope present on the fungal cell surface. When F1 and F2 were subjected to SDS-PAGE, transblotted and stained with enzyme-conjugated mAb AF1 or Con A, most of the antibody or lectin bound to high molecular mass (>200 kDa) polydisperse material, some of which was present in F2 (as in the starting GMP extract) but absent in F1. This difference was also observed in PAS-stained gels of the two fractions. The F2, but not the F1, constituent was as active as the unfractionated GMP extract in inducing lymphoproliferation, production of the cytokines interleukin-2 and interferon- γ , and generation of cytotoxicity against a natural-killer-sensitive target cell line (K562). These immunomodulatory properties were, like those possessed by GMP, protease-sensitive and heat-stable. Treatment of PBMC cultures with a modulatory anti-T-cell receptor antibody abolished the lymphoproliferation induced by GMP and F2 but not that induced by phytohaemagglutinin, showing that the mannoprotein materials of *C. albicans* acted through interaction with the antigen receptor complex.

Introduction

Candida albicans is a human commensal and opportunistic pathogen which possesses several distinctive properties of a potent biological response modifier (Cassone *et al.*, 1987; Domer *et al.*, 1988). It is important to investigate these properties in detail, as their comprehension may offer additional insight into the nature of the *Candida*-host relationship, both in normal subjects and in patients with candidosis. As an example of biological response modifier properties of this fungus, the activation of non-MHC-restricted antitumour cytotoxic effectors is well established (Weinberg & Hibbs,

1979; Cassone *et al.*, 1981; Marconi *et al.*, 1983, 1985; Wojdani & Ghoneum, 1987; Djeu *et al.*, 1988; Scaringi *et al.*, 1988, 1990). It is also clear that at least part of these immunomodulatory effects of *C. albicans* can be induced by mannoprotein constituents from its cell wall. In addition to glucan, a mannoprotein extract was highly effective in activating natural killer cells and macrophages in mouse peritoneal cavity (Scaringi *et al.*, 1988), or in stimulating lymphoproliferation and non-MHC restricted cytotoxicity in *in vitro*-cultured human peripheral blood mononuclear cells (PBMC) (Ausiello *et al.*, 1986, 1989). Many other immunomodulatory effects, including the induction of immunosuppressive activities against both B and T cell-mediated responses, have been also ascribed to mannoproteins or 'mannan' of *C. albicans* (Carrow & Domer, 1985; Cuff *et al.*, 1986, 1989; Durandy *et al.*, 1987; Podzorski *et al.*, 1989, 1990). In view of the complexity of the biological response

Abbreviations: Con A, concanavalin A; E:T, effector:target; GMP, glucomannoprotein; IFN, interferon; IL-2, interleukin-2; mAb, monoclonal antibody; MHC, major histocompatibility complex; PAS, periodic acid-Schiff; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin.

modifier properties expressed by candidal mannoproteins, including both immunoenhancing and immunosuppressive effects (Domer *et al.*, 1986, 1987), it is important to examine distinct mannoprotein fractions separately for their immunological effects. This paper focuses on chromatographically separated, chemically and antigenically defined mannoprotein fractions that are responsible for the overall immunoenhancing activity of the crude mannoprotein extract of *C. albicans*.

Methods

Preparation of cell wall mannoprotein extract of *C. albicans*. The cell wall glucomannoprotein extract (GMP) was obtained from a clinical isolate of *C. albicans* (strain BP) as previously reported (Ausiello *et al.*, 1986). Briefly, washed yeast cells were autoclaved in a neutral buffer (140 °C, 2 h), and the cell lysate was precipitated in cold ethanol and extracted with chloroform/butanol (5:1, v/v). The aqueous phase was extensively dialysed against water and lyophilized.

Chromatographic fractionation of the extract. The GMP extract was subjected to ion-exchange chromatography essentially as described elsewhere (Cassone, 1989; Torosantucci *et al.*, 1990). Briefly, 200 mg GMP, dissolved in water, was loaded on a 241 × 2.6 cm column of DEAE-Sephadex A-50 (Pharmacia) in 0.05 M-Tris/HCl pH 7.5. After a prolonged wash with the equilibrating buffer to remove unbound material, retained fractions were eluted with two consecutive linear gradients of increasing NaCl concentration (0.1–0.4 M and 0.4–2.0 M, respectively). The eluate was collected in 7 ml fractions which were assayed for polysaccharide content and absorbance at 260 nm. Fractions corresponding to polysaccharide peaks were pooled, extensively dialysed against water and lyophilized. The final yield was about 82% of the loaded polysaccharide. Each fraction was subjected to the limulus lysate gelification test (PBI, Milan) to detect endotoxin (lipopolysaccharide) contamination.

Analytical determinations. Protein concentrations were determined by Lowry's method, using bovine serum albumin (BSA) as the standard. Total polysaccharide was determined by the phenol/sulphuric acid method according to Dubois *et al.* (1956), using glucose as the standard. 'Mannan' was defined as Fehling-precipitable, saccharide-positive material (see also Torosantucci *et al.*, 1990). Because of the very low percentage of glucan in GMP (Ausiello *et al.*, 1986; Scaringi *et al.*, 1988), this mannan closely paralleled the amount of mannose determined by gas chromatography (Scaringi *et al.*, 1988). Total phosphorus was assayed by the method of Chen *et al.* (1956).

SDS-PAGE analysis. Samples containing an appropriate amount of crude mannoprotein extract or chromatographic fractions were subjected to SDS-PAGE according to Laemmli (1970), using either 10% or 5–10% (w/v) gradient acrylamide polymers as separating gels and 4.5% or 3.5% acrylamide slabs as stacking gels. The gels were either stained with periodic acid-Schiff (PAS) reagent or transblotted onto nitrocellulose paper and stained with the monoclonal antibody (mAb) AF1 (see below) followed by phosphatase-conjugated, affinity-purified goat anti-mouse-IgM antibodies (Sigma), as previously described (Torosantucci *et al.*, 1990). Alternatively, the blots were stained with peroxidase-conjugated concanavalin A (Con A), as follows. The nitrocellulose sheets were saturated by incubation for 1 h at room temperature with 3% (w/v) BSA in 0.01 M-Tris/HCl buffer, pH 7.5, containing 1 mM-CaCl₂ and 1 mM-MnCl₂ (TCM), then incubated overnight at 4 °C with peroxidase-conjugated Con A (Sigma) at a concentration of 0.052 U ml⁻¹, in TCM. After extensive washings

with TCM, the bound lectin was detected by treating the sheets with 20 mM-Tris/HCl, pH 7.5, containing NaCl (500 mM), 4-chloro-1-naphthol (0.5 mg ml⁻¹), methanol (17%, v/v) and hydrogen peroxide (0.017%), as the peroxidase substrate.

Reactivity with antibodies. The crude mannoprotein extract and its chromatographic fractions were assayed for their reactivity with anti-*Candida* serum and with mAb AF1, directed against an oligosaccharide epitope of the candidal cell surface (Cassone *et al.*, 1988; Torosantucci *et al.*, 1990). An indirect ELISA and an ELISA-inhibition assay were used, as reported previously (Ausiello *et al.*, 1986; Scaringi *et al.*, 1988; Cassone *et al.*, 1988). The polyclonal serum was generated in rabbits by immunization with four subcutaneous administrations, at weekly intervals, of 2×10^7 glutaraldehyde-inactivated cells of *C. albicans* strain BP (Ausiello *et al.*, 1986). The candidal cells were mixed 1:1 with Freund's complete adjuvant (the first two injections) or with saline (the second two injections), in a total injected volume of 1 ml. Blood was taken by cardiopuncture and the serum heated at 65 °C for 30 min before reaction with appropriate antigen. mAb AF1 was purified by protein A-MAPS II buffer affinity chromatography, as detailed elsewhere (Mariani *et al.*, 1989). Both the polyclonal serum and mAb AF1 had a titre >1:100000 in an ELISA reaction using 5 µg GMP ml⁻¹ as coating antigen.

Mannoprotein treatments. Samples (10 mg) of GMP or fractions were dissolved in 5 ml phosphate-buffered saline (PBS) containing 0.5 mg pronase (grade A, Sigma) and the mixture was incubated overnight at 37 °C. The pronase was then precipitated by heating at 100 °C for 10 min; the mixture was centrifuged, the precipitate discarded and the supernatant dialysed extensively against PBS. Control samples (heat-treated material) were suspended in pronase-free PBS, left overnight at 37 °C, then heated and processed as described above. The proteolytic treatment consistently removed more than 80% of protein material from both the initial extract and the purified fraction, whereas the heat treatment alone left the chemical composition of treated materials almost unaltered.

PBMC preparation and proliferation assays. PBMC of normal healthy adult donors were isolated, cultured and stimulated with phytohaemagglutinin (PHA), anti-CD3 mAbs, GMP and mannoprotein constituents, essentially as described previously (Ausiello *et al.*, 1986, 1989). Briefly, 96-well trays containing 10^6 PBMC ml⁻¹ and the established dose of each inducer or reagent (as indicated in single experiments) in complete RPMI medium containing 10% (v/v) pooled human AB serum were incubated for different times under 5% (v/v) CO₂ at 37 °C. Each batch of human serum used did not contain anti-*Candida* antibodies detectable by a slide agglutination test. Cell proliferation was measured as [³H]thymidine incorporation. In selected experiments, PBMC were pretreated for 24 h with the non-mitogenic anti-CD3 mAb CBT3-M, then washed and incubated with or without stimulants as indicated in each experiment.

Generation of cytotoxic effector cells and measurement of cytotoxicity. Freshly separated PBMC, at a concentration of 10^6 cells ml⁻¹, were incubated for 7 d in flat-bottomed 24-microwell trays (Nunc, Denmark) in 1 ml volumes in the presence of putative cytotoxicity inducers. Cytotoxicity generated in these cultures was assayed against the human erythroblastoid cell line K562. Target cells were labelled in a 1 h incubation at 37 °C with 150 µCi of a Na₂⁵¹CrO₄ solution [specific activity 298 mCi ml⁻¹ (11 GBq ml⁻¹), NEN-Dupont] in a final volume of 0.4 ml. After labelling and washing, cells were resuspended in complete RPMI medium and re-incubated for 30 min at 37 °C. Cytotoxicity assays were performed in round-bottomed 96-microwell trays, as reported elsewhere (Ausiello *et al.*, 1989), using different effector:target (E:T) ratios. The percentage of specific lysis was calculated by the formula $100 \times [(experimental\ c.p.m. - spontaneous\ c.p.m.) / (total\ c.p.m. - spontaneous\ c.p.m.)]$.

ous c.p.m.)/(maximum c.p.m. minus spontaneous c.p.m.)). Results are expressed as means of triplicate samples for each E:T ratio. The standard errors of the mean, usually less than 2.0%, are not shown.

Cytokine determination. Supernatants of PBMC cultures were harvested at selected intervals and assayed for interleukin-2 (IL-2) using the IL-2-dependent murine T cell line CTLL-2 (Gillis *et al.*, 1971). The IL-2 concentration was expressed in units (U) ml⁻¹ as described by Gillis *et al.* (1971), using a recombinant IL-2 preparation (kindly provided by Dr G. Garotta, Roche, Basel, Switzerland) as an internal reference standard. In the same supernatants, the titre of interferon (IFN) was determined by a previously described assay for the inhibition of Sindbis virus haemagglutinin yield after a single growth cycle (Stanton *et al.*, 1987). IFN activity was expressed as international units (IU). It was characterized as IFN- γ by the use of polyclonal monospecific antisera against IFN- α , - β or - γ , as previously reported (Spagnoli *et al.*, 1985).

Results

Chromatographic separation, and chemical and antigenic characterization of mannoprotein extract

In keeping with previous reports (Cassone, 1989; Torosantucci *et al.*, 1990), three main, well-separated components were obtained by ion-exchange chromatography of the GMP extract. One fraction (F1), representing about 15% of the material applied to the column, was not retained by the resin, being eluted before application of the NaCl gradient. A second fraction (F2), eluting at 0.1–0.2 M-NaCl, was the most abundantly recovered (47.6% of the material applied to the column). A third distinct constituent (F5), with strong absorbance at 260 nm and relatively high phosphorus content, was eluted at the highest salt concentration. It represented about 18% of the starting material. Minor, poorly resolved saccharide materials (F3 and F4) were eluted between 0.4 and 0.6 M-NaCl, but, because of their paucity, they were not analysed further or investigated for their immunomodulatory effects.

Fractions F1 and F4 were all strongly reactive in ELISA with the polyclonal anti-*Candida* serum whereas fraction F5 reacted weakly with it. Fractions F1 to F4 also contained a sugar epitope (probably an oligomanno-

side) recognized by mAb AF1 (Cassone *et al.*, 1988). Fraction F5 did not react with this mAb. The serological specificity of the fractions was maintained after heat treatment and deproteinization (see below and Cassone *et al.*, 1988).

Chemically (Table 1), both the resin-unbound (F1) and the low-salt-eluted (F2) fractions were composed predominantly of mannoprotein with a low but detectable phosphorus content. That mannose was the predominant sugar constituent of F1 and F2 was confirmed by gas-liquid chromatography (93.6% and 91.9% mannose in F2 and F1, respectively). Protein was more abundant in F2 than in F1 (5.0% vs 2.6%). Fraction F5 contained relatively little polysaccharide (22%). Its strong absorbance at 260 nm and high phosphorus content (relative to the other fractions) suggested that nucleic acid was a major component. In addition, fraction F5 was the only one to give a (slightly) positive limulus lysate test, indicating the presence of some contaminating endotoxin. Thus, this fraction was used as an impurity and negative control in further experiments (see below).

When fractions F1 and F2 were run in SDS-PAGE at 10% gel concentration and immunoblotted with mAb AF1 or stained with PAS, each showed polydisperse material of high molecular mass (>200 kDa) which barely penetrated the separating gel (Fig. 1*a, b*). F2 was more disperse than F1, and contained materials smearing towards lower molecular mass regions, as evident in the PAS-stained gel. Some of this material was present in GMP but absent in F1. Low molecular mass (<30 kDa) PAS-staining material was present only in the GMP extract (Fig. 1*a*).

More details were observed when the fractions were run in 5–10% acrylamide gradient gels, transblotted and reacted with Con A-peroxidase conjugate (Fig. 2). Although maintaining their pronounced polydispersity, the extracts were resolved as discrete, though poorly defined, bands or regions. As in PAS-stained gels (Fig. 1*a*) one marked difference between fractions F1 and F2 was the absence in the former of part of the component in the high molecular mass region close to the origin of the

Table 1. *Crude chemical composition of GMP and its main subfractions*

Values are expressed in μg per 100 μg of lyophilized material of a batch of GMP preparation (GMP-8) that was used for all experiments reported in this paper. Minor batch-to-batch variations in the chemical composition were noted in equally active preparations of GMP.

Material	Total polysaccharides	Mannan	Protein	Phosphorus
Unfractionated GMP	72.5	68.8	8.4	2.7
F1	>95.0	>95.0	2.6	0.01
F2	>95.0	>90.0	5.0	0.03
F5	22.0	1.5	2.5	3.3

Fig. 1. SDS-PAGE (10% gel concentration) of GMP and chromatographic fractions F1 and F2, stained with PAS (a) or immunoblotted with mAb AF1 (b). In (a), each lane contained 1 mg of material; in (b), each lane contained 20 μ g of material.

Fig. 2. SDS-PAGE (5–10% gradient gel) of GMP and chromatographic fractions F1 and F2, run at doses of 200 μ g (a) or 100 μ g (b), transblotted and stained with peroxidase-conjugated Con A. The experimental conditions are described in Methods. The arrows are explained in the text.

Fig. 3. Kinetics of PBMC proliferation after stimulation with GMP (○) and its chromatographic fractions F1 (●), F2 (▲) and F5 (■), all used at 50 μ g ml⁻¹ in each PBMC culture. □, Untreated PBMC culture from the same donor (control).

gel (arrows in Fig. 2). This component was present in both GMP and F2. Three main bands (two with a molecular mass above 110 kDa and one in a broad region around 65 kDa) were present in both fractions. A few well-defined mannoprotein bands were also present (at 35–36 kDa) in the two fractions (Fig. 2).

Lymphoproliferation and generation of anti-K562 cytotoxicity by mannoprotein fractions

The two predominant mannoprotein fractions (F1 and F2) were compared to the crude mannoprotein extract (GMP) and to the impure fraction (F5) for their capacity to induce lymphoproliferation, production of the cytokines IL-2 and IFN- γ , and cytotoxicity in human PBMC from normal donors. In a series of preliminary experiments, PBMC were exposed to different doses of *Candida*-derived material and cultured for 10 d to examine the kinetics of immunostimulation. Fig. 3 shows a typical experiments where PBMC were incubated for 10 d with 50 μ g ml⁻¹ of each material. Proliferation of GMP-stimulated PBMC was detectable on day 5 and reached a plateau on days 7–10, confirming previous reports (Ausiello *et al.*, 1986). Of the three fractions examined, only F2 induced PBMC proliferation, similar, in extent and time-course, to that induced by the unfractionated extract; F1 and F5 showed minimal, if any, activity (Fig. 3). In the same experiment, cytotoxicity, as measured by lysis of K562 tumour target cells, was also significantly induced by candidal material on days 7–10 (data not shown). For this reason, day 7 was selected to measure lymphoproliferation and K562 lysis by *Candida* fractions.

Table 2 shows the results obtained with PBMC from three normal subjects (different from the one used in the

Table 2. Proliferation and generation of cytotoxic activity in PBMC stimulated for 7 d with GMP and its fractions

Antigen	Dose ($\mu\text{g ml}^{-1}$)	Proliferation ($10^{-3} \times [^3\text{H}]$ thymidine incorporation, c.p.m., $\pm$ SD)			Cytotoxic activity* (% specific lysis) at E:T		
		Donor 1	Donor 2	Donor 3	50:1	20:1	10:1
None	—	0.91 $\pm$ 0.09	0.58 $\pm$ 0.05	2.5 $\pm$ 0.8	24.0	19.6	7.9
Unfractionated	50	6.3 $\pm$ 1.0	10.7 $\pm$ 1.6	55.7 $\pm$ 6.7	95.7†	94.7†	79.4†
GMP	5	7.8 $\pm$ 3.4	16.5 $\pm$ 0.8	38.3 $\pm$ 7.7	95.5†	78.1†	65.8†
	0.5	12.8 $\pm$ 2.9	13.5 $\pm$ 3.1	—	—	—	—
F1	50	1.9 $\pm$ 0.3	1.8 $\pm$ 0.3	4.8 $\pm$ 0.9	79.0†	55.0‡	37.5‡
	5	1.2 $\pm$ 0.1	1.7 $\pm$ 0.2	5.2 $\pm$ 2.6	64.0†	39.0	28.2
	0.5	0.8 $\pm$ 0.2	0.9 $\pm$ 0.5	—	—	—	—
F2	50	6.9 $\pm$ 3.7	14.4 $\pm$ 3.9	52.1 $\pm$ 8.6	98.2†	85.8†	76.4†
	5	3.3 $\pm$ 0.8	7.7 $\pm$ 2.0	21.7 $\pm$ 4.7	83.2†	81.9†	66.5†
	0.5	1.6 $\pm$ 0.7	1.6 $\pm$ 0.3	15.3 $\pm$ 2.1	—	—	—
F5	50	1.2 $\pm$ 0.4	2.4 $\pm$ 0.6	5.2 $\pm$ 2.0	39.9	36.0	8.6
	5	0.9 $\pm$ 0.2	0.8 $\pm$ 0.2	2.4 $\pm$ 0.7	—	—	—
	0.5	0.2 $\pm$ 0.02	0.7 $\pm$ 0.3	—	—	—	—

* Measured as lysis of K562 cells in 4 h ^{51}Cr -release assay using the cells from donor 3 as lytic effectors.

† $P < 0.01$ (Student's *t* test), as compared to the control, unstimulated culture.

‡ $P < 0.05$, as above.

experiment shown in Fig. 3) stimulated with GMP and its fractions at doses of 0.5, 5 and 50 $\mu\text{g ml}^{-1}$. F2 was the only chromatographic fraction to induce significant, dose-dependent, lymphoproliferation. Both F1 and F5 were inactive, even at 50 $\mu\text{g ml}^{-1}$. The generation of anti-K562 cytotoxicity was manifested in F2-treated PBMC, but some lytic activity was also generated in F1-treated cultures, especially when assayed at the highest E:T ratio (Table 2). Fraction F5 did not stimulate lymphoproliferation or cytotoxicity.

Cytokine production

The production of the cytokines IL-2 and IFN- γ , which are particularly important in the proliferation and cytotoxicity of PBMC (Balkwill & Burke, 1989), was also investigated in PBMC stimulated by candidal fractions. In one typical experiment (out of four performed), shown in Table 3, significant IL-2 and IFN production were detected with the same materials (GMP and F2) which stimulated lymphoproliferation. With cells from the donor reported in Table 3, an amount of IFN- γ (300 IU per ml of culture) equalling that given by incubation of PBMC with 100 U of recombinant IL-2 ml^{-1} was measured. In keeping with its inability to stimulate lymphoproliferation, fraction F1 did not induce the production of appreciable quantities of IL-2. However, this fraction was able to induce some IFN production (Table 3, Fig. 4). The kinetics of IL-2 and IFN production after stimulation with *Candida*-derived materials is shown in Fig. 4 (a, b), which refers to a typical experiment performed with PBMC of a donor different from that used in the experiment reported in Table 3.

Both GMP and F2 induced cytokine production, which was maximal on day 2 for IL-2 and on day 7 for IFN (Fig. 4 and data not shown). For this particular PBMC donor, 500 IU of IFN were produced by F2-stimulated PBMC. Although there were quantitative differences between donors in the degree of GMP- and F2-stimulation of cytokine production, the qualitative pattern was comparable and reproducible in all seven donors examined. The IFN was characterized as IFN- γ by the use of sera specific for IFN- α , - β or - γ (Spagnoli *et al.*, 1985).

The antigenic nature of lymphoproliferation induced by mannoprotein fractions

We previously demonstrated that GMP-induced lymphoproliferation was antigenic in nature (Ausiello *et al.*, 1986). To extend this finding to the F2 mannoprotein fraction, we used an anti-CD3 mAb (CBT3-M) which is unable to induce lymphoproliferation but, through binding to the CD3Ti complex, induces TCR modulation, thus inhibiting antigen-specific proliferation (Alcover *et al.*, 1987). The results of an experiment with two donors are summarized in Table 4, which shows that proliferation induced by either GMP or its F2 fraction was virtually abolished in CBT3-M-treated PBMC, while PHA-stimulated lymphoproliferation was unaffected.

Effect of protease or heat treatment on the immunological activity of GMP and fraction F2

As both GMP and its active immunomodulatory constituent F2 had protein moieties, it was of interest to

Table 3. Proliferation and cytokine production in cultures of human PBMC, unstimulated or stimulated with candidal materials and human recombinant interleukin-2 (rIL-2)

PBMC were cultured without or with the indicated stimulant, at the desired concentration, at 37 °C. Proliferation, measured by [³H]thymidine incorporation, was assessed on day 7 of the culture; IL-2 and IFN- γ production were assayed on days 2 and 7, respectively (see also Fig. 4).

Stimulant	Dose ($\mu\text{g ml}^{-1}$)	PBMC proliferation ($10^{-3} \times \text{c.p.m.}, \pm \text{SD}$)	IL-2 (U ml^{-1})	IFN- γ (IU ml^{-1})
None	—	4.5 ± 1.3	0.8	10
GMP	50	43.8 ± 1.9	5.9*	100*
	5	30.0 ± 2.3	2.1	30
F1	50	5.5 ± 1.4	1.0	100*
	5	4.0 ± 2.5	0.8	20
F2	50	81.5 ± 12.9	5.2*	300*
	5	21.2 ± 8.7	1.7	10
F5	50	5.6 ± 1.8	1.4	20
	5	2.9 ± 0.7	0.9	10
rIL-2	100†	41.3 ± 1.0	—	300*

* Denotes a statistically significant ($P < 0.01$) difference with respect to the unstimulated control, as assessed by the Student's *t* test.

† Units ml^{-1} .

Fig. 4. Kinetics of production of IL-2 (a) and IFN (b) in PBMC cultures from a healthy donor unstimulated (□), or stimulated with GMP (○) or its fractions F2 (▲), F1 (●) or F5 (■). Each stimulant was used at a concentration of 50 μg per ml of PBMC culture.

examine whether hydrolysis or protein modification would result in a loss of immunological activity. Materials were therefore treated with pronase, and, as the experimental procedure for pronase treatment included a non-specific precipitation in boiling water, controls containing only GMP and buffer were similarly treated at 100 °C. The data from these experiments are shown in Table 5 (two donors for lymphoproliferation and one for cytotoxicity generation). Protein degradation largely, if not completely, abolished both lymphoproliferation and cytotoxicity induction by both the unfractionated extract and fraction F2. Only at the highest E:T ratio (50:1) was some residual cytotoxicity generated by heat- and pronase-treated *Candida*-derived material. Simple heat treatment of GMP and F2 did not abolish their capacity to stimulate lymphoproliferation and cytotoxicity generation (Table 5). Moreover, a control experiment performed with a pronase solution, heated as above, and incubated with PBMC, did not affect either the lymphoproliferation or cytotoxicity induction (data not shown).

Discussion

To understand better the nature of the host-*Candida* relationship, we have studied the antigenic and immunomodulatory activities of a mannoprotein extract, GMP, of *C. albicans* (Spagnoli *et al.*, 1985; Ausiello *et al.*, 1986, 1989; Scaringi *et al.*, 1988). It is well known that crude 'mannan' or mannoprotein of fungal cell wall contains a

Table 4. Effect of mAb CBT3-M on PBMC proliferation induced by GMP, fraction F2, or PHA

PBMC, untreated or pretreated (24 h) with MAb CBT3-M ($10 \mu\text{g ml}^{-1}$), were stimulated with the indicated candidal material (each at $50 \mu\text{g ml}^{-1}$) or PHA [1:100 dilution of a commercial preparation (HA-15; Wellcome): see Ausiello *et al.* (1986)]. Proliferation was measured on day 7 or 3 for *Candida*- or PHA-stimulated cultures, respectively.

Stimulant	PBMC proliferation ($10^{-3} \times [^3\text{H}]$ thymidine incorporation, c.p.m., $\pm$ SD)					
	Donor 1			Donor 2		
	Untreated	CBT3-M-treated	P*	Untreated	CBT3-M-treated	P*
None	0.3 $\pm$ 0.07	1.2 $\pm$ 0.09	—	1.05 $\pm$ 0.2	0.8 $\pm$ 0.08	—
GMP	11.8 $\pm$ 2.7	1.5 $\pm$ 0.6	<0.01	8.61 $\pm$ 1.2	0.5 $\pm$ 0.2	<0.01
F1	2.6 $\pm$ 0.3	1.9 $\pm$ 0.4	NS	—	—	—
F2	25.1 $\pm$ 1.0	2.6 $\pm$ 0.2	<0.01	9.4 $\pm$ 2.5	1.0 $\pm$ 0.2	<0.01
F5	2.1 $\pm$ 2.8	1.3 $\pm$ 0.2	NS	—	—	—
PHA	75.4 $\pm$ 5.6	81.7 $\pm$ 6.3	NS	108.4 $\pm$ 9.3	92.3 $\pm$ 9.1	NS

* P values comparing CBT3-M-treated with antibody-untreated cultures. NS, Not significant.

Table 5. Lymphoproliferation and cytotoxicity induction by immunologically active fractions after proteolytic treatment

Antigen	Dose ($\mu\text{g ml}^{-1}$)	PBMC proliferation ($10^{-3} \times [^3\text{H}]$ thymidine incorporation, c.p.m., $\pm$ SD)				Cytotoxic activity (% specific lysis) at E:T)		
		Donor 1	P*	Donor 2	P*	50:1	20:1	10:1
None	—	0.9 $\pm$ 0.09	—	2.5 $\pm$ 0.8	—	35.0	29.7	26.1
Unfractionated	50	7.0 $\pm$ 2.4	—	55.7 $\pm$ 6.7	—	98.7	94.7	79.4
GMP	5	9.4 $\pm$ 2.1	—	33.3 $\pm$ 7.7	—	95.5	88.1	85.8
Pronase- and heat-treated GMP	50	1.3 $\pm$ 0.3	<0.01	6.1 $\pm$ 3.6	<0.01	67.5†	44.0†	31.7†
Heat-treated GMP	5	1.5 $\pm$ 0.3	<0.01	3.2 $\pm$ 1.5	<0.01	55.0†	27.6†	13.7†
F2	50	6.2 $\pm$ 2.0	NS	50.1 $\pm$ 5.5	NS	—	—	—
	5	8.8 $\pm$ 2.2	NS	28.7 $\pm$ 6.3	NS	—	—	—
	5	6.9 $\pm$ 3.7	—	52.1 $\pm$ 8.6	—	100	85.8	76.4
Pronase- and heat-treated F2	50	3.3 $\pm$ 0.8	—	21.7 $\pm$ 4.7	—	83.2	81.9	76.9
	5	1.3 $\pm$ 0.3	<0.01	5.6 $\pm$ 2.7	<0.01	56.0†	37.0†	22.3†
	5	1.5 $\pm$ 0.3	<0.01	6.2 $\pm$ 2.8	<0.01	50.4†	37.0†	31.3†
Heat-treated F2	50	6.4 $\pm$ 1.2	NS	49.5 $\pm$ 2.9	NS	—	—	—

* P values comparing treated versus the respective untreated antigen preparation. NS, Not significant.

† P < 0.01 vs PBMC cultures induced by untreated GMP or F2, respectively (Student's *t* test).

‡ P < 0.05 vs PBMC cultures induced by untreated GMP or F2, respectively (Student's *t* test).

heterogeneous mixture of constituents with relatively small differences in gross chemistry but markedly different immunological properties. Domer *et al.* (1988) and Cassone (1989) have recently reviewed the evidence for the varied effects of mannoprotein constituents of *C. albicans* on the immune system. Remarkably, both immunoenhancing and immunosuppressive effects have been ascribed to this material (Carrow & Domer, 1985; Domer *et al.*, 1986, 1987; Scaringi *et al.*, 1988; Ausiello *et al.*, 1989; Cuff *et al.*, 1989), although it is not clear whether these apparently opposite effects are caused by different constituents or even by a single constituent, used in different ways or concentrations, or both. For all these reasons, the separation and identification of active mannoprotein constituents is important. Our chromatographic procedure, similar to that previously used by Domer *et al.* (1986) on a different mannan preparation,

separated GMP into three well-resolved main peaks (F1, F2, F5), with evidence of poorly resolved minor constituents. One of the main fractions (F5) appeared to consist of a heterogeneous material, heavily contaminated by nucleoprotein. The other two fractions, F1 and F2, were essentially mannoproteins, similar in chemical composition to those obtained by others (Domer *et al.*, 1986; Shibata *et al.*, 1985, 1986). These latter authors used a bulk phosphomannoprotein preparation obtained by digestion of whole cells with a 1,3- β -glucanase and precipitation with Cetavlon, a procedure likely to give mostly undegraded material. With respect to this preparation, our F1 and F2 fractions had a higher percentage of protein and a lower phosphorus content. Other authors (Podzorski *et al.*, 1989, 1990) have recently prepared a 'mannan' extract by fractional precipitation with Cetavlon of autoclaved *Candida* cells. Since the

material contained about 10% protein, this 'mannan' is indeed a mannoprotein extract comparable to our GMP extract or fraction F2 in gross chemistry and immunological activity (Podzorski *et al.*, 1990).

Despite the similarities in chemical composition, there must be important, immunologically relevant, molecular differences between the two mannoprotein fractions separated here, as only F2 had the whole spectrum of immunological activities shown by the crude extract. Some immunomodulatory activities, in particular cytotoxicity and IFN production, were also manifested in PBMC cultures of some donors treated with the highest dose of F1 fraction, and it is possible either that fractions F1 and F2 partly share the inductive constituent or that different constituents may bring about the same phenomena, although with quite different efficiency.

When examined by SDS-PAGE, fractions F1 and F2 both showed polydisperse mannoprotein material with electrophoretically distinct complexes of different molecular mass. Some differences between the molecular profiles of the two fractions were noticed, in particular concerning a high molecular mass, poorly separated constituent which was absent in F1 but present in both F2 and GMP. It is not known whether this or some other undetected difference is relevant to the lack or the substantial reduction of PBMC-proliferation and IL-2-production-inducing abilities of the F1 fraction. Immunologically active, mannan preparations used by others have not been examined by SDS-PAGE, so a comparison with our data is not possible. It is known, however, that mannan obtained by Fehling precipitation suffers from alkaline degradation and loss of protein (Nakajima & Ballou, 1974; Shibata *et al.*, 1986), and we have shown here that the protein moiety is essential for mannoprotein to be active as an immunostimulator. Interestingly, protein was also essential in the DTH-phenomenon elicited by *Candida* glycoprotein in experimental animals (Carrow & Domer, 1985).

We previously presented data suggesting that *Candida* mannoproteins did not act as mitogens but rather as specific antigens (Ausiello *et al.*, 1986). The experiments using anti-CD3 antibodies, showing almost total inhibition of lymphoproliferation induced by GMP or F2, but not of that induced by PHA, reinforce previous evidence. The F2 fraction was not contaminated by limulus-lysate-detectable lipopolysaccharide, while the F5 fraction, which was slightly positive, was substantially inactive in our model. This seems to rule out the possibility that some of the observed effects, in particular those concerning the cytotoxicity induction, could be due to endotoxin. The antigenic nature of *Candida*-derived mannoprotein-stimulated lymphoproliferation of human PBMC is also confirmed by recent data of Podzorski *et al.* (1990).

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