

Anticandidal Activity and Interleukin-1 β and Interleukin-6 Production by Polymorphonuclear Leukocytes Are Preserved in Subjects with AIDS

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Received 2 November 1992/Accepted 19 February 1993

Polymorphonuclear granulocytes (PMN; or neutrophils) from uninfected or human immunodeficiency virus-infected subjects were tested for their ability to inhibit growth of *Candida albicans* and produce interleukin-1 β (IL-1 β) and IL-6 in vitro. It was seen that PMN from AIDS (Centers for Disease Control stage IV) patients expressed equal if not greater anticandidal activity compared with the activity expressed by neutrophils from all other subjects examined. On exposure to granulocyte macrophage-colony-stimulating factor or to a mannoprotein constituent (MP-F2) from *C. albicans* itself, PMN from AIDS patients showed enhanced antifungal activity and production of remarkable quantities of IL-1 β and IL-6. These findings suggest that the functional abilities of PMN to inhibit *Candida* growth and secrete relevant proinflammatory and immunomodulatory cytokines are intrinsically preserved in AIDS patients.

Patients with AIDS suffer from immunologic defects which almost invariably lead to increased susceptibility to opportunistic pathogens, among which *Candida albicans* has a prominent role (5, 7). Various forms of mucosal candidiasis are indeed common in these patients, but deep-seated candidiasis is rare and seems to occur only in AIDS patients with major risk factors for disseminated disease such as neutropenia or high-dose steroids (6, 7). As polymorphonuclear leukocytes (PMN; or neutrophils), possibly with the participation of monocyte-macrophages and antibodies to specific constituents of *C. albicans* (2, 10), play a critical role in the defense against disseminated candidiasis, their anticandidal activity should not be expected to be grossly impaired in AIDS subjects. Nonetheless, neutrophil dysfunctions leading to defective candidacidal activity in AIDS subjects have been repeatedly reported (4).

PMN can be largely potentiated in their antimicrobial activity by stimulants like cytokines and bacterial lipopolysaccharide (LPS). Interestingly, mannoprotein constituents of *C. albicans* itself (MP-F2) are as powerful as LPS and granulocyte macrophage-colony-stimulating factor (GM-CSF) in potentiating the anti-*Candida* activity of PMN from healthy subjects in vitro (12). On this basis, we reexamined the in vitro anticandidal activity by unstimulated or MP-F2- or GM-CSF-stimulated neutrophils from subjects at different stages of human immunodeficiency virus (HIV) infection. We also assayed unstimulated and stimulated neutrophils for their ability to produce cytokines as markers of their activation.

This study was carried out with outpatients visiting a clinical immunology section of a department of infectious diseases, during a period of 6 months. All adult subjects who gave informed consent to donate blood for the specific aims of the study were eligible for inclusion. All subjects taking antimycotics in the 2 weeks preceding blood collection were

excluded. Thus, we enrolled 44 adult subjects (10 women and 34 men). The 28 with HIV infection were subgrouped according to their clinical stage (13), with 10 subjects in Centers for Disease Control (CDC) stage II or III and 18 subjects in CDC stage IV (C₁ or C₂). The mean numbers (\pm standard deviations) of CD4⁺ peripheral blood lymphocytes were 456 (\pm 225) and 201 (\pm 140) for subjects at CDC stages II and III and at CDC stage IV, respectively. The absolute neutrophil counts were (3,160 \pm 1,120) \times 10⁶/liter and (2,570 \pm 1,140) \times 10⁶/liter (means \pm standard deviations) for the two groups of subjects, respectively. In particular, none of the AIDS patients was frankly neutropenic, as defined by PMN counts of less than 1,000 \times 10⁶/liter. Of the 16 HIV-negative subjects, 9 were healthy individuals not belonging to any recognized risk category for HIV infection, while 7 were drug addicts (4 individuals) or had HIV-positive sexual partners (3 individuals). All CDC stage IV patients were under treatment with zidovudine for at least 6 months. None of the HIV-positive subjects was affected by oropharyngeal, vaginal, or esophageal candidiasis at the time of the study. The mean age and male/female ratio did not differ between HIV-negative and HIV-positive subjects, as well as between CDC stage II and III and CDC stage IV subjects.

The strain of *C. albicans* used as the target of neutrophil activity and the cell wall mannoprotein fractions (MP-F2) used throughout this study were described elsewhere (12, 13). In particular, MP-F2 was obtained by ion-exchange chromatographic separation of a mannoprotein-rich extract (GMP) from *C. albicans*, previously characterized for its immunogenic properties (12, 13). MP-F2 was devoid of any contaminating LPS (12).

All procedures in this study were performed with endotoxin-free media and reagents to avoid nonspecific activation of neutrophils. Heparinized blood samples were diluted 1:2 in RPMI 1640 and layered on 10 ml of Lymphoprep (Nyegaard, Oslo, Norway). After centrifugation (30 min at 400 \times g) at room temperature, the PMN layer lying on the surface of the erythrocyte pellet was collected and the erythrocytes

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TABLE 1. Stimulation of neutrophil anticandidal activity by mannoprotein of *C. albicans* or GM-CSF

HIV infection stage	No. of subjects	PMN anticandidal activity ^a (mean ± SE inhibition units)		
		Unstimulated	GM-CSF stimulated	MP-F2 stimulated
HIV negative	16	180.1 ± 33.9 ^b	491.4 ± 121.4 ^c	455.4 ± 60.4 ^d
CDC stages II and III	10	137.4 ± 28.2 ^e	538.2 ± 151.3 ^f	423.4 ± 91.8 ^g
CDC stage IV	18	359.0 ± 74.3 ^h	998.7 ± 229.5 ⁱ	895.3 ± 187.8 ^j

^a Expressed as the number of PMN causing 20% *Candida* growth inhibition (see text). The statistical significance of the differences in the anticandidal activity was the same when the activity was calculated in terms of 50% inhibition of *Candida* growth (not shown in the table). $P < 0.05$ (pooled Student's *t* test), *b* or *e* versus *h*; $P < 0.05$ (pooled Student's *t* test), *d* versus *j*; $P < 0.01$ (paired Student's *t* test, *b* versus *c* or *d*, *e* versus *f* or *g*, and *h* versus *i* or *j*. All other comparisons gave statistically nonsignificant differences ($P > 0.05$).

were lysed by hypotonic shock with sterile distilled water for 30 s at room temperature. The cells were washed twice in RPMI 1640 before they were readjusted to the desired density. Cytocentrifuged, Giemsa-stained preparations showed these cells to be >99% PMN by morphology.

The anti-*Candida* activity of neutrophils was determined by a radiolabel microassay to measure growth inhibition of *C. albicans*, as described elsewhere (3, 12). Briefly, various numbers of neutrophils in RPMI 1640 containing 2% heat-inactivated fetal calf serum (FCS), 5 mmol of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer per liter, 2 mmol of L-glutamine per liter, 10 U of penicillin per ml, and 100 µg of streptomycin per ml (FCS medium) were added to triplicate wells of a 96-well flat-bottomed microplate. For PMN activation, 10 µg of MP-F2 per ml or 10³ U of GM-CSF per ml was added to the wells and the neutrophils were incubated for 30 min at 37°C. Then *C. albicans* yeast cells, suspended in 25 µl of FCS medium, were added to each well to result in effector/target (E/T) ratios of 60/1, 30/1, and 15/1. *Candida* cells were also added to empty wells to serve as controls. After incubation at 37°C for 18 h under 5% CO₂, 10 µCi of [³H]glucose (D-[5,6-³H]glucose) specific activity, 66.9 Ci/mmol; Dupont de Nemours & Co., NEN Products, Boston, Mass.) per ml in sterile water was added. After an additional 3 h of incubation at 37°C, the cells were harvested and the mean incorporation of triplicate cultures was determined with a β-counter (Be-taplate; LKB, Bromma, Sweden) (the standard error was usually within 5% of the mean). The percent growth inhibition of *Candida* cells was given by the following: (cpm of *Candida* cells alone – cpm of PMN/*Candida* cell)/cpm of *Candida* cells alone × 100.

Inhibition units were calculated and expressed as inhibition units 10⁷ effector cells on the basis of the various E/T ratios used. One inhibition unit is defined as the number of effector cells causing 20% growth inhibition of 500 *Candida* cells. The growth inhibition was linear over the range of E/T ratios tested, and the average values of the slopes of the curves relating E/T ratios to growth inhibition were not significantly different among the different groups of subjects studied.

Interleukin-1β (IL-1β) and IL-6 production was assayed by enzyme-linked immunosorbent assay (Quantikine) in supernatants of neutrophils (2.5 × 10⁶/ml of FCS medium in 24-well Costar plates) after 18 h of incubation in the presence or absence of MP-F2 (10 µg/ml).

Since the anticandidal activity of neutrophils from HIV-negative normal subjects and that from HIV-negative subjects belonging to risk categories were almost equal, all data from HIV-negative subjects have been cumulated in a single group in Table 1.

There was no statistically significant difference between the anticandidal activity of neutrophils from this group of subjects and that of PMN from CDC stage II and III subjects. Unstimulated PMN from AIDS patients (CDC stage IV) showed not only an intact *Candida* growth-inhibitory potential but also an activity globally higher than that expressed by the unstimulated neutrophils from all other subjects. Table 1 also shows that the neutrophils were strongly enhanced in their anticandidal activity by GM-CSF and MP-F2. The potentiation observed in all HIV-positive subjects fell in the same range (about 2.5 times the value of unstimulated neutrophils) as that achieved by GM-CSF or MP-F2 in PMN from HIV-negative subjects. Thus, the highest anticandidal activity was shown by GM-CSF- or MP-F2-stimulated PMN from AIDS patients (998 ± 229 and 895 ± 187 inhibition units, respectively). The enhancement of the anticandidal activity of PMN by the mannoprotein constituent of *C. albicans* was, in all subject groups, statistically not different from that obtained with GM-CSF. There was no statistically significant difference in neutrophil counts between AIDS patients and all other HIV-infected subjects (see above), and no relationship could be found between inhibition unit levels and neutrophil count.

Figure 1 shows the individual values of anticandidal activity in unstimulated and MP-F2-stimulated PMN. Despite the large individual variations, mostly evident in AIDS patients, in all subjects the stimulated neutrophils had higher inhibition unit levels than the unstimulated counterpart at each stage of HIV infection. It is noteworthy that (i) three of the five CDC stage IV subjects showing extreme PMN anti-*Candida* activity values (>1,000 inhibition units) had fewer than 10 × 10⁶ CD4⁺ cells per liter and (ii) one of these subjects also had the lowest absolute PMN count, i.e., 1,060 × 10⁶/liter.

The neutrophils from the subjects at different stages of HIV infection who donated sufficient quantities of blood were assayed for IL-1β and IL-6 production at the 18th hour of incubation in the presence or in the absence of MP-F2. Table 2 shows that the neutrophils produced the two cytokines under MP-F2 stimulation and that the cytokine level was similar in all study groups.

Previous reports on the anticandidal activity of neutrophils in AIDS patients have led to contrasting conclusions because different groups of subjects, different PMN functions, and different anticandidal assays have been studied. In particular, no data on the effect of candidal components themselves on PMN activity have been presented. In their comprehensive review, Ellis et al. (4) noticed that binding of *C. albicans* for phagocytosis was impaired in PMN from AIDS-related complex or AIDS patients. However, the neutrophils had increased expression of a functionally rele-

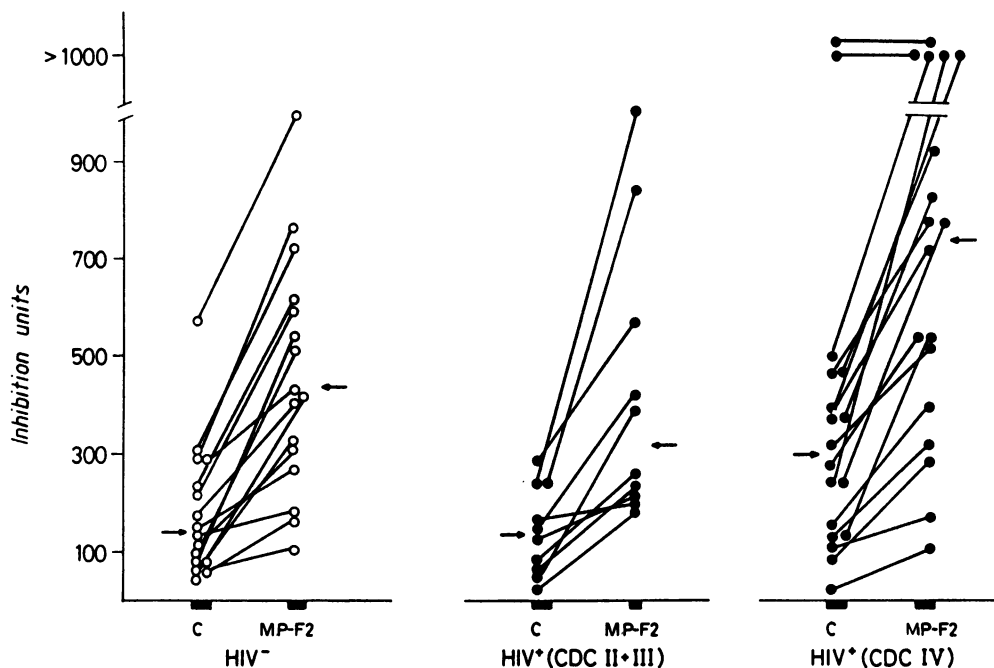


FIG. 1. Anticandidal activity of PMN from uninfected or HIV-infected subjects. The neutrophils were either unstimulated (C) or stimulated with the mannoprotein activator of *C. albicans* (MP-F2). The arrows point to median inhibition unit values.

vant surface component such as the iC3b receptor. Other authors attributed to heroin abuse rather than to HIV infection the decreased ability of AIDS-related complex or AIDS patients' PMN to ingest and kill *C. albicans* (8).

Contrasting results have also been reported concerning the phagocytosis and killing of *Staphylococcus aureus* by PMN from AIDS patients (1, 4, 11). In general, the results suggesting dysfunctions in the anticandidal activity of the neutrophils from AIDS patients are not in keeping with current clinical evidence that even terminally ill, profoundly immunodepressed AIDS patients, though suffering from extensive mucosal candidiasis, are not particularly prone to invasive disease.

The radiolabel microassay of PMN anticandidal activity used here cumulates candidastatic with candidacidal effects and is equally effective in monitoring the growth inhibition (and/or killing) of both ingestible yeast particles and un ingestible hyphal filaments (3, 12). This avoids the strong bias inevitable in the enumeration of CFU when multicellular hyphae are present, as is the case in all tissue culture media. The conditions of our assay may also better reflect the in vivo situation, in which the anti-*Candida* effectors are

challenged by both yeast and mycelial elements and both candidacidal and candidastatic mechanisms are likely to be expressed by the neutrophils (14).

Thus, we obtained clear-cut evidence that (i) neutrophils from HIV-infected subjects are as effective in inhibiting growth of *C. albicans* in vitro as those from HIV-uninfected subjects; (ii) PMN from highly immunodepressed AIDS patients may nonetheless be potent anti-*Candida* effectors in vitro, capable of responding positively to a physiological stimulant (GM-CSF) or an active component of *C. albicans* itself; and (iii) PMN from HIV-infected subjects are capable of synthesizing relevant proinflammatory and immunomodulatory cytokines, such as IL-1 β and IL-6, upon stimulation with mannoprotein of *C. albicans*, exactly as occurs with neutrophils from healthy subjects (12). Interestingly, the baseline *Candida* growth inhibitory potential of PMN from AIDS patients averaged a higher value compared with that of PMN from non-AIDS-patient subjects. Although statistically significant, this difference should be taken with caution because of the particularly large spread in the anticandidal response of PMN from AIDS patients (Fig. 1) and the relatively low number of subjects studied in each category.

TABLE 2. IL-1 and IL-6 production by PMN from different groups of uninfected or HIV-infected subjects

Subject group	Cytokine production ^a (mean \pm SE [pg/ml]) (no. of subjects examined)			
	IL-1		IL-6	
	Unstimulated	MP-F2 stimulated	Unstimulated	MP-F2 stimulated
HIV negative	18.9 \pm 7.0 (11)	151.5 \pm 81.3 (10)	6.8 \pm 3.4 (9)	430.9 \pm 119.4 (9)
CDC stages II and III	3.8 \pm 2.5 (8)	127.2 \pm 84.5 (8)	1.2 (5) ^b	407.3 \pm 141.4 (5)
CDC stage IV	5.3 \pm 3.1 (9)	160.0 \pm 90.4 (9)	29.6 \pm 14.9 (9)	339.0 \pm 156.5 (9)

^a There were no statistically significant differences in cytokine production among the three subject groups, whatever the stimulus used. There were no statistically significant differences in age and male/female ratio between the subjects tested for PMN cytokine production and the untested ones.

^b Only one subject gave a measurable amount of cytokine.

Nonetheless, it is of interest that the anticandidal activity of unstimulated PMN from AIDS patients approached to that of GM-CSF- or MP-F2-stimulated PMN from uninfected subjects (Table 1). This raises the possibility that the AIDS patients studied here had somewhat preactivated PMN *in vivo*, because of either circulating cytokines, as, for instance, tumor necrosis factor alpha (9), or concurrent infections. As *Candida* mannoprotein is detectable in the blood of several AIDS patients, with or without clinical signs of mucosal candidiasis (2a), this constituent itself could act *in vivo*. These possibilities are currently being evaluated. Overall, the data shown here strongly suggest that the AIDS patient neutrophil is *per se* still an efficient anticandidal effector, matching the clinical evidence that patients with AIDS are not at risk of deep-seated candidiasis unless they become severely neutropenic. Our data also raise the theoretical possibility that neutrophils from AIDS patients are able to respond *in vivo* to activation signals, such as those furnished by GM-CSF, other cytokines, and microbial immunomodulators (LPS and mannoprotein), thus exerting more effective antimicrobial activity.

This work was supported by a grant from Ministero della Sanità, Istituto Superiore di Sanità, contract 720/X (National AIDS Project).

We are greatly indebted to A. M. Marella for help in preparing the manuscript.

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