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# Opsonophagocytosis and Intracellular Killing Activity of Neutrophils in Patients with Human Immunodeficiency Virus Infection

**Kalaya Tachavanich[1], Kovit Pattanapanyasat[2], Suttipant Sarasombath[3], Surapol Suwannagool[4] and Vinai Suvattee[1]**

**SUMMARY** **In this study, neutrophils isolated from asymptomatic HIV positive individuals, patients with AIDS-related complex (ARC), ARC patients receiving zidovudine (AZT) and full-blown AIDS patients were assayed for their opsonophagocytic and intracellular killing activities. Progressively decreasing opsonophagocytosis of *C. albicans* by neutrophils correlated with increasing severity of the disease in all groups of HIV infected individuals, as compared to neutrophils isolated from healthy controls. The intracellular killing of *C. albicans* by neutrophils of asymptomatic and ARC patients did not differ significantly from controls. Neutrophils of ARC patients receiving AZT and AIDS patients showed a slightly decreased killing activity in comparison to that of neutrophils from healthy controls.**

A hallmark of progression of HIV infection to acquired immune deficiency syndrome (AIDS) is opportunistic infections with microorganisms that rarely cause disease in individuals with normal immune systems.[1] The high incidence of opportunistic infection in AIDS patients indicates inadequacy of host defense against invading microorganisms. Neutrophils which constitute the first line of defense can phagocytose many microorganisms and kill them intracellularly.[2] These antimicrobial activities can be enhanced by cytokines (interferon-$\gamma$) derived from T lymphocytes. HIV infection is known to be associated with T lymphocyte abnormalities including a reduction in the number of $CD4^+$ cells,[3,4] a diminished proliferative response to antigens and mitogens[5,6] and a decrease of the release of cytokines upon antigen stimulation.[7] Thus, these T lymphocyte abnormalities should influence the functions of neutrophils in HIV infected patients.

It is not clear whether intrinsic defects of neutrophil function in HIV infected patients are responsible for the observed diminished antimicrobial activities, since both normal and impaired phagocytosis and intracellular killing have been previously reported for phagocytic cells from HIV infected patients.[8,12] These contradictory results have been attributed to types of *Candida* species, stages of diseases, methods of investigation, or whether the patients were parenteral drug users.

The objectives of this study are to use flow cytometry to evaluate the opsonophagocytosis and intracellular killing activities of neutrophils from HIV-infected patients from different stages of diseases and who are not parenteral drug users.

## MATERIALS AND METHODS

### Subjects

Thirty patients with asymptomatic infection and PGL (persistent generalized lymphadenopathy), 30 patients with ARC (AIDS-Related Complex), 27 patients with ARC receiving zidovudine (AZT 500 mg/day for at least 4 weeks) and 19 patients with full-blown AIDS

From the [1] Department of Pediatrics, [2] Office of Research and Development, [3] Department of Immunology, [4] Department of Preventive Medicine: Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Correspondence : Kovit Pattanapanyasat

receiving zidovudine (AZT 500 mg/day for at least 4 weeks) and 19 patients with full-blown AIDS were included in this study. None of these patients received steroids or other immunomodulatory drugs at the time of this study. None were parenteral drug users. They were under the supervision of the Infectious Disease Clinic and Counselling, Siriraj Hospital, Mahidol University. Seventy healthy blood donors from Siriraj Hospital Blood Bank, negative for anti-HIV antibodies, were included as the control subjects. There were 35 males and 35 females (aged between 19 and 56 years). All subjects were given fully informed consent. A microcell counter (Sysmex F-500 Kobe, Japan) was used to obtain hematologic data for each individual. Flow cytometic immunophenotyping of CD4 and CD8 lymphocytes was performed using monoclonal antibodies to CD4 and CD8 (Leu 3 and Leu 2, Becton Dickinson, San Jose, USA). Results are expressed as absolute $CD4^+$ and $CD8^+$ cells which obtained from white blood cell count, lymphocyte differential (percent lymphocytes in white blood cell count) and the preent $CD4^+$ or $CD8^+$ cells.

**Separation of PMN cells**

Approximately 5 ml peripheral venous blood was collected and anticoagulated with heparin 2.5 IU/ml. Polymorphonuclear cells (PMN) were purified by dextran sedimentation[13] and centrifugation over Ficoll-hypaque gradient (density 1.077 g/ml, Sigma Chemical Company, St Louis, MO, USA). PMN cells were then washed once with sterile phosphate buffered saline (PBS) and resuspended in their own plasma. The resulting cell preparations consisted of > 90% viable PMN cells as assessed by tryptan blue dye, and > 97% purity of PMN cells as assessed by Wright's staining. PMN cells for opsonophagocytosis assay were labelled with phycoerythrin (PE) conjugated anti-CD13 monoclonal antibody (Mab) MY-7 (Coulter Electronics, Krefeld, Germany) at a final antibody concentration of 5 μg/ml for 30 minutes on ice.[14] The labelled PMN cells were washed once with cold PBS at 300 × g for 5 minutes and resuspended in their own plasma.

**Preparation of *C. albicans***

A modified technique for the labelling of *C. albicans,* as described by Martin and Bhakdi[14] was followed. *C. albicans* were cultured overnight (10–16 hours) at 37°C in 4 ml brain heart infusion broth (GIBCO Laboratories, Madison, WI, USA). After the culture period, *C. albicans* were centrifuged at 1,800 × g for 2 minutes, and the pellet was resuspended in 1 ml of 0.9% NaCl, containing 30 μl of 0.29 mM bis-carboxyethyl-carboxyfluorescein pentaacetoxymethylester (BCECF-AM, Calbiochem-Behring Corp, La Jolla, Calif.) to make a final concentration of 10 μM and incubated for 30 minutes at 37°C. During the incubation period, BCECF-AM diffused into *C. albicans* and was cleaved by *C. albicans* cytoplasmic esterase to yield the green fluorescence. Only viable cells can retain the membrane-impermeable final product, bis-carboxyethyl-carboxyfluorescein (BCECF).[15] The labelled *C. albicans* were washed three times with 0.9% NaCl at 1,800 × g for 2 minutes to remove free dye and resuspended at a final concentration of 1:1 ratio of *C. albicans* to PMN in 0.9% NaCl.

**Opsonophagocytosis assay**

In this assay, labelled PMN cells were incubated with labelled *C. albicans* at 37°C for 0, 10, 20 or 60 minutes in a horizontal shaking water bath. After various incubation periods, the reaction was stopped by addition of 500 μl of 0.1% paraformaldehyde buffer (Fisher Scientific Company, New Jersey, USA) and the results were analysed with a FACScan (Becton Dickinson) flow cytometer.

**Intracellular killing assay**

Unlabelled PMN cells were incubated with labelled *C. albicans* (ratio 1:1) at 37°C. A control sample contained only *C. albicans* and plasma. After a 60 minute incubation period, 100 μl of 1% (w/v) solution of deoxycholate (DOC); Fluka AG, Buchs, Switzerland) and 250 μl of distilled water were added to the control and test sample tubes for 5 minutes and 3 minutes at 37°C, respectively. DOC solubilizes blood cells and liberates viable and nonviable *C. albicans* from PMN cells. Both viable green fluorescence and nonviable non-fluorescence *C. albicans* were counted using flow cytometer.

**Flow cytometric analysis**

Analysis of both opsonophagocytosis and intracellular killing assay was performed using a FACScan equipped with a 15 mW argon ion laser tuned at 488 nm. In opsonophagocytosis assay, two-color flow cytometric analysis of red and green fluorescence of both MY-7 positive PMN and BCECF labelled *C. albicans* were used. Logarithmic green fluorescence was observed through a 530/30 nm band pass filter. Simultaneously, red fluorescence was detected through a 585/42 nm band pass filter. For phagocytic killing assay, only green fluorescence was used. *C. albicans* were gated on the basis of their forward light scatter (FSC) and side scatter (SSC) using linear scale.

For each sample, 5,000 PMN and 2,000 *C. albicans* were analysed for their fluorescence intensity using LYSIS software (Becton Dickinson). Opsonophagocytosis results were presented as double color dot plot. For phagocytic killing, a percentage of positive viable *C. albicans* was determined by histogram analysis.

### Statistical analysis

The result was analysed by Mann-Whitney U test. All data were expressed as mean ± SEM. P values < 0.05 were considered statistically significant.

## RESULTS

### Laboratory findings in HIV infected patients

Table 1 summarizes the hematologic data including total white blood cell, differential counts, and the $CD4^+$ and $CD8^+$ T lymphocyte numbers of the healthy controls and HIV infected patients. Asymptomatic HIV infected patients tended to have higher total white blood cell counts, absolute neutrophil counts and absolute lymphocyte count than did patients with ARC receiving AZT and patients with AIDS, but were not significantly different from those in patients with untreated ARC. The mean $CD4^+$ T lymphocyte count and $CD4^+$:$CD8^+$ ratio were lower in all patient groups than in healthy controls. The number of $CD4^+$ T lymphocyte and the $CD4^+$:$CD8^+$ ratio gradually decreased along the clinical spectrum from asymptomatic patients to ARC patients and AIDS patients, but were not significantly different between ARC patients and ARC patients receiving AZT. The mean $CD8^+$ T lymphocyte counts in all patient groups were significantly higher than in healthy control.

**Table 1.** Total white blood cell count, $CD4^+$ and $CD8^+$ T lymphocyte counts of asymptomatic HIV infected individuals, ARC patients, ARC patients receiving AZT, AIDS patients and healthy controls.

| | No. of patients | Age (years) | Total white blood cell ($mm^3$) | Neutrophil ($mm^3$) | Lymphocyte ($mm^3$) | CD4 ($mm^3$) | CD8 ($mm^3$) | CD4/CD8 ($mm^3$) |
|---|---|---|---|---|---|---|---|---|
| Asymptomatic | 30 | 29.0 ± 1.5* | 7,727 ± 372** | 4,772 ± 365** | 2,330 ± 123 | 446 ± 41* | 1,161 ± 79* | 0.45 ± 0.06* |
| ARC | 30 | 33.0 ± 1.8 | 7,973 ± 645** | 4,926 ± 444** | 2,337 ± 251** | 237 ± 39* | 1,311 ± 163* | 0.19 ± 0.03* |
| ARC + AZT | 27 | 36.0 ± 1.9 | 5,859 ± 381** | 3,618 ± 244 | 1,912 ± 175* | 248 ± 26* | 1,133 ± 127* | 0.25 ± 0.03* |
| AIDS | 19 | 34.0 ± 1.4 | 5,642 ± 540** | 3,414 ± 476** | 1,747 ± 164* | 142 ± 38* | 1,141 ± 152* | 0.13 ± 0.03* |
| Healthy controls | 70 | 36.0 ± 1.3 | 6,514 ± 200 | 3,853 ± 156 | 2,383 ± 112 | 861 ± 48 | 750 ± 35 | 1.20 ± 0.05 |

*$p < 0.001$ vs. healthy controls,
**$p < 0.05$ vs. healthy controls.

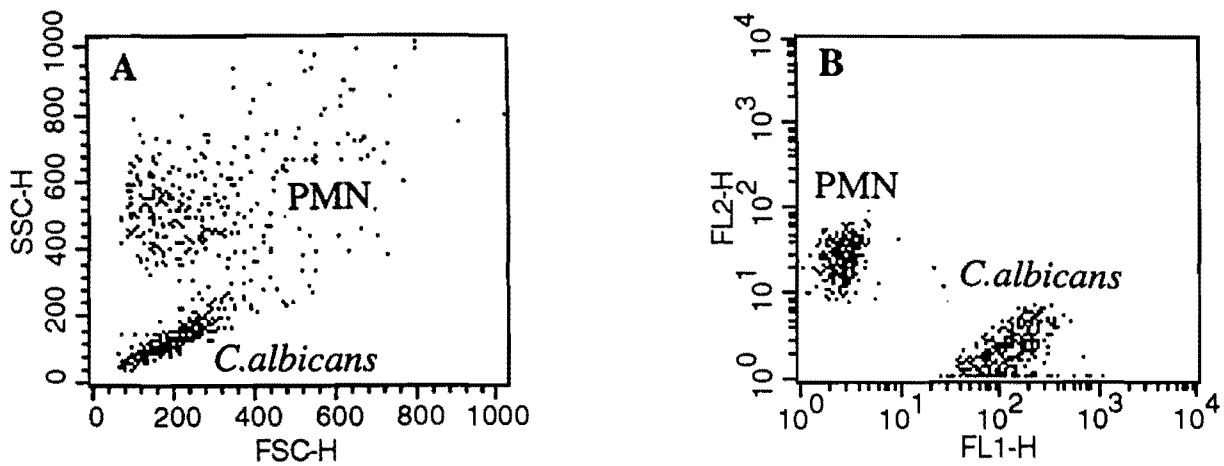


**Fig. 1** Characteristics of labelled *C. albicans* and labelled PMN cells. **A** shows the distribution pattern of cells analyzed according to forward scatter (FSC) and side scatter (SSC). **B** shows the relative green fluorescence (F11; BCECF) and orange fluorescence (F12; phycoerythrin-labelled anti-CD13).

**Flow cytometric analysis of opsonophagocytosis**

After the labelling of PMN cells and *C. albicans* with fluorochrome-conjugate MY-7 and BCECF, respectively, these two cell populations were incubated together at different time periods. At 0 minute, PMN cells can be accurately distinguished from *C. albicans* population using both FSC/SSC (Fig. 1A) and fluorescence (Fig. 1B). To quantiate the percentage of *C. albicans* phagocytosed by PMN cells at various incubation time, a live gate was applied to PMN cells according to their SSC and PE (F12) fluorescence (Fig. 2). Fig. 3 shows both two-color dot-plots and F11 (BCECF) histogram gated of PMN cells after 0, 10, 20 and 60 minutes of coincubation with *C. albicans*. The shift towards green (F11) fluorescence of PMN cell population increased with time. Flow cytometric results of opsonophagocytosis of *C. albicans* by PMN cells from asymptomatic HIV infected patients, ARC patients, ARC patients receiving AZT and AIDS patients were compared with those from healthy controls at 10, 20 and 60 minutes (Table 2). Control PMN cells showed maximal opsonophagocytosis binding after 20 minutes and these binding activities remained constant over the following 60 minutes of incubation time. PMN opsonophagocytosis and binding of *C. albicans* was significantly less ($P < 0.05$) in patients with ARC, ARC patients receiving AZT and AIDS patients as compared with those from healthy controls. No statistically significant differences were recorded between patients with ARC, ARC patients receiving AZT and AIDS patients at 10, 20 and 60 minutes incubation time.

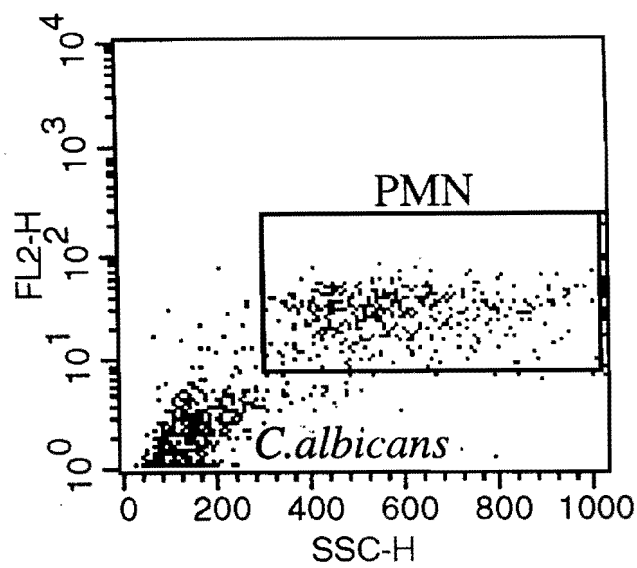


**Fig. 2.** Demonstration of live-gate setting for determining the percentage of PMN cells associating with *C. albicans*. The live-gate is set around the My-7 (CD13)-labelled population of cells (PMN), identified on the basis of orange fluorescence (F12) and the typical granularity (SSC).

**Table 2.** Summary of *C. albicans* phagocytosed by PMN and the intracellular killing activity of PMN cells (mean ± SEM) in HIV infected patients and healthy controls.

| Subjects | No. | % *C. albicans* ingested PMN | | | % killing |
|---|---|---|---|---|---|
| | | 10 min. | 20 min. | 60 min. | 60 min. |
| Asymptomatic | 30 | 31.44 ±2.16* | 43.48 ±2.26* | 60.27 ±1.87 | 31.46 ±1.45 |
| ARC | 30 | 25.36 ±1.96* | 38.63 ±2.45* | 55.04 ±2.64** | 27.28 ±2.20 |
| ARC+AZT | 27 | 24.23 ±2.07* | 33.95 ±2.71* | 47.75 ±2.45* | 24.97 ±2.29** |
| AIDS | 19 | 22.51 ±3.00* | 35.98 ±3.48* | 51.05 ±3.62* | 20.37 ±2.19* |
| Control | 70 | 45.13 ±1.32 | 53.82 ±1.17 | 61.20 ±1.08 | 29.80 ±0.95 |

*$p < 0.001$ vs. healthy controls.
**$p < 0.05$ vs. healthy controls.

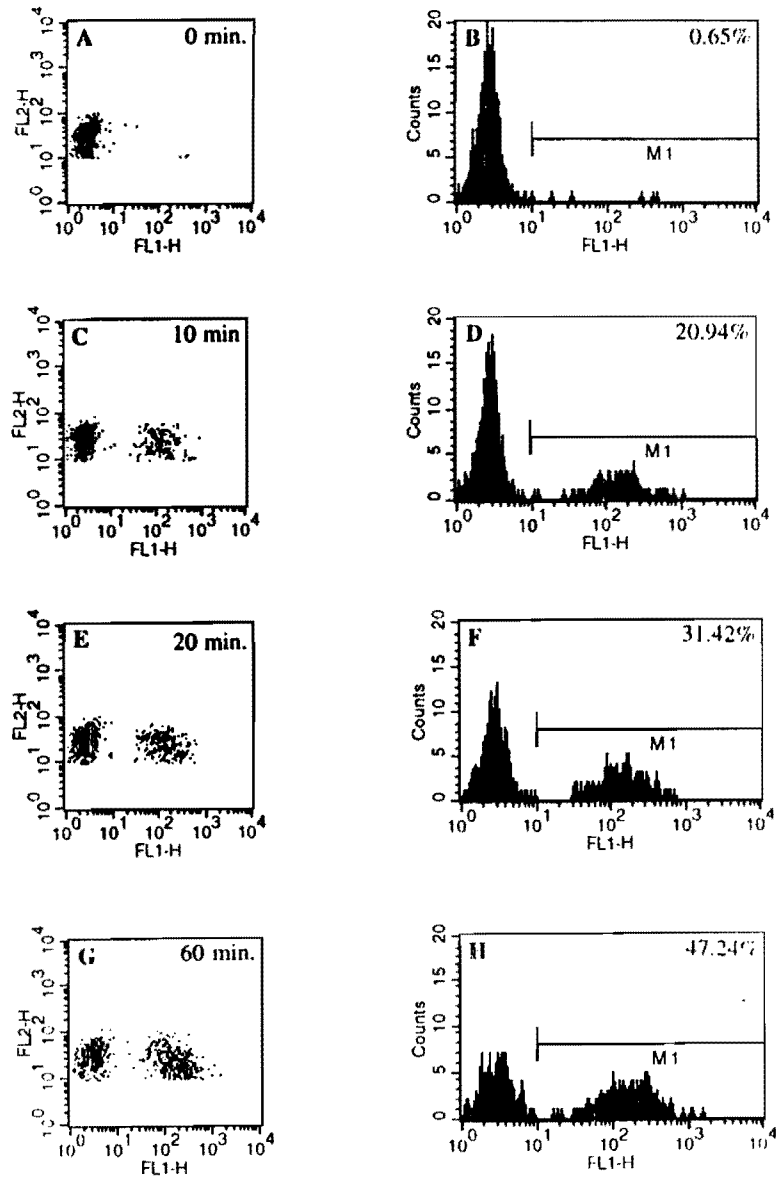


**Fig. 3.** Flow cytometric analysis of opsonophagocytosis assay. The distribution patterns of gated PMN cells with and without asociated *C. albicans* organisms according to F11 (green) and F12 (orange) after incubation at 37°C for 0, 10, 20 and 60 minutes **(A, C, E, G)**. The percentage of phagocytes exhibiting bright green fluorescence at the given time points is depicted **(B, D, F, H,)**. The marker (solid line) defining the commencement of positive green fluoescence was set as shown.

A significant impairment of opsonophagocytosis was detected in asymptomatic HIV infected patients when compared to healthy controls ($P \leq 0.05$) at 10 and 20 minutes but not at 60 minutes incubation time.

**Flow cytometric analysis of intracellular killing**

After incubation of non-labelled PMN cells and BCECF-labelled *C. albicans* for 60 minutes at 37°C, PMN cells were solubilized with DOC and both viable and non-viable *C. albicans* were liberated from the PMN cells. To quantitate the percentage of killing, *C. albicans* were gated according to their FSC and SSC (Figs. 4A, 4D). Viable and nonviable *C. albicans* were distinguishable by their separation in determined SSC versus F11 (Figs. 4B, 4E). Table 2 summarizes the percent killing of *C. albicans* by PMN cells from the 5 groups. The percentages of *C. albicans* killed intracellularly by PMN cells from patients of the different groups show great inter-individual variations. Intracellular killing was significantly lower ($P \leq 0.05$) in patients with ARC receiving AZT and in AIDS patients than in healthy controls, but asymptomatic and ARC patients were not significantly different from those in control groups. No statistically significant differences were recorded between ARC patients and ARC patients receiving AZT. The impairment of killing of *C. albicans* by PMN cells was greater in patients with AIDS than in patients with ARC.

## DISCUSSION

The ineffective immunity in patients with HIV infection contributes to severe and widespread secondary infections and unrestricted growth by certain tumors. A profound depression of cell-mediated immunity has been described by several authors, especially with respected to T-lymphocyte numbers and function.[3,4] However, the

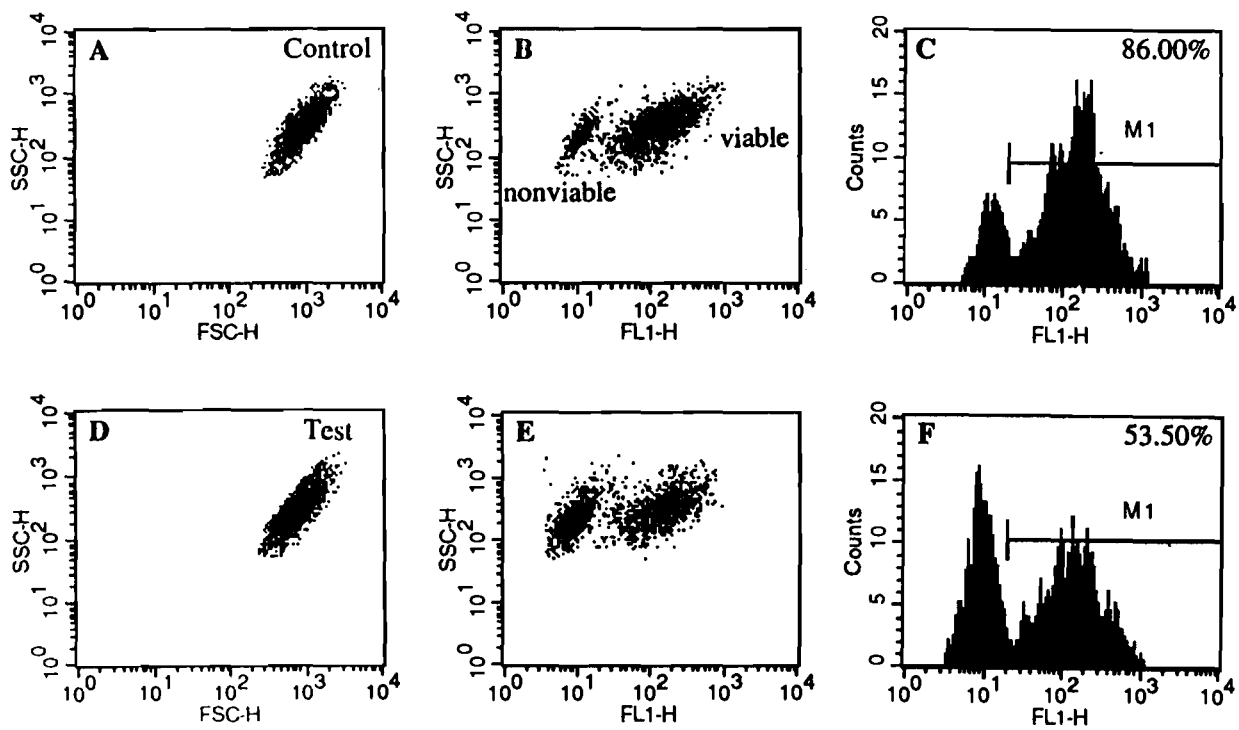


Fig. 4. Flow cytometric analysis of intracellular killing assay. Demonstration of *C. albicans* in control and test groups accor ding to FSC and SSC (**A, D**). SSC versus F11 (**B, E**). Fluorescence intensity is reduced in nonviable *C. albicans. Whereas the majority of C. albicans* incubated with plasma (control tube) were fluorescent, the numbers of non-fluorescent particles increased in the test samples Histogram of F11 (**C, F**). The percentage of fluorescent particles was calculated as shown in the histograms. Percent specific killing was estimated as the percentage of fluorescent particles in control sample minus the percentage of flurescent particle in test sample x 100, divided by the percentage of fluorescent particle in control sample.

functional defects of neutrophils in patient infected with HIV are still not well elucidated.

Neutrophils play an important role in the defense against bacterial infections and certain fungal infections. Literature on quantitative neutrophil abnormalities in asymptomatic HIV infected individuals, patients with ARC and AIDS patients is scarce. Neutropenia has recently been reported to occur in AIDS.[16] In one large study,[17] neutropenia occurred rarely with seroconversion but was more commonly observed (up to 40% of patients) with progression to AIDS. Klaassen *et al.*[18] found that anti-neutrophil cytoplasmic autoantibodies appeared in the asymptomatic stages and their prevalence increased in the symptomatic stage. In our study, neutropenia was present in patients with ARC receiving AZT and in AIDS patients but not in asymptomatic patients and patients with ARC. Both the direct myelosuppressive effect of AZT[19,20] and the presence of autoantibodies to neutrophils that have been described in the late stages of HIV infection may contribute to the observed neutropenia.

Our study demonstrated decreased opsonophagocytosis of *C. albicans* in neutrophils from all groups of HIV infected patients when compared to healthy controls. As clinical progression through ARC to AIDS occurred, a corresponding decrease in opsonophagocytosis was observed. However, the effectiveness of intracellular killing in asymptomatic and ARC patients was not different from that of controls. A slightly decreased intracellular killing was observed in AIDS patients and ARC patients receiving AZT.

The defects in opsonophagocytosis and intracellular killing of neutrophils in HIV infected patients may result from several factors. A decrease in the binding of opsonized microorganisms to phagocytes might be a result of a decreased expression of FcR and/or complement receptors on the surface of phagocytes

in these patients.[21,22] Another possible explanation is the defective signal transduction induced by HIV proteins (as reported for $CD4^+$ T lymphocytes,[23,24]) which accounts for the impaired phagocytosis of microorganism by affecting the assembly and disassembly of the actin filaments involved in the formation of pseudopodia to engulf microorganisms.

Intracellular killing by PMN cells directly depends on oxidative and nonoxidative metabolic products (*eg*, phagosome-lysome fusion, or secretion of microbicidal products in response to the particular organisms). The defects in killing activity by phagocytes of HIV infected patients have been ascribed to deficiency of the myeloperoxidase system;[25,27] impairment of phagolysosomal fusion,[28] and depressed oxidative metabolism with a positive relationship between $O_2$-production and absolute $CD4^+$ lymphocyte count.[29] It is possible that plasma-membrane receptor-mediated activation of the oxidative burst may be impaired. Additionally, serum from HIV-infected patients with low $CD4^+$ T lymphocyte counts have been reported to be able to suppress the killing activity of normal PMN cells compared with serum from normal donors.[8] Viral particles or synthetic viral envelope peptides have also been found to directly impair the function of phagocytes.[30]

Other factors, such as relative changes in lymphokines and monokines released by HIV-infected lymphocytes and monocytes may be reponsible for impairing PMN function at different stages. In this regard, the cytokines of the Th2 type of immune response (*eg* interleukin-4 or -10) may suppress the function of phagocytes in HIV-infected patients.[31,32] Alternatively, the apparent suppressive effect of serum from HIV-infected patients may be related to impaired production of other cytokines belonging to the Th1 type of immune response. Th1 cytokine responses which predominate in healthy asymptomatic individuals including the production of interferon-$\gamma$ has been known to cause up-regulation of the antifungi activity of normal phagocytes. The deficiency of interferon-$\gamma$ in symptomatic patients may contribute to the dysfunction of theirs phagocytes.[33]

In conclusion, this study suggests the existence of defects in PMN cell functions of HIV infected patients. These defects may increase as the disease progresses towards AIDS. The progression of these defects corresponds with the progressive decrease of T lymphocyte count and of the $CD4^+/CD8^+$ ratio, implying that $CD4^+$ T lymphocyte deficiency in these patients correlates with and may contribute to their abnormal PMN functions.

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