Impaired phagocytosis of *Staphylococcus aureus* by granulocytes and monocytes of AIDS patients

O. POS, A. STEVENHAGEN, P. L. MEENHORST*, F. P. KROON & R. VAN FURTH *Department of Infectious Diseases, University Hospital, Leiden, and *Department of Internal Medicine, Slotervaart Hospital, Amsterdam, The Netherlands*

SUMMARY

In the present study the microbicidal activities of granulocytes and monocytes from AIDS patients (CDC group IV) were assessed and compared with those of healthy controls. The phagocytosis and intracellular killing of *Staphylococcus aureus* by patient and control cells were measured using a method in which the rate of intracellular killing can be assessed independently of the rate of phagocytosis. Both granulocytes and monocytes of AIDS patients showed a decreased phagocytosis of *S. aureus* in comparison to phagocytes of healthy individuals. The rates of intracellular killing of *S. aureus* by granulocytes and monocytes did not differ significantly between these patients with late-stage HIV infection and controls.

Keywords AIDS *Staphylococcus aureus* monocytes granulocytes phagocytosis intracellular killing

INTRODUCTION

One of the major characteristics of AIDS is the high incidence of opportunistic infections, indicating that the host defense against invading micro-organisms is inadequate. The most important effector cells of the antimicrobial defense mechanism are granulocytes, monocytes, and macrophages. These cells can phagocytose micro-organisms and kill them intracellularly [1]; all of these anti-microbial activities can be enhanced by cytokines. AIDS is associated with T lymphocyte abnormalities including a reduction in the number of CD4+cells [2,3], a diminished proliferative response to antigens and mitogens [4,5], and a decrease of the release of cytokines upon antigen stimulation [6]. The occurrence of opportunistic infections by intracellular micro-organisms is due to insufficient activation of phagocytic cells by cytokines.

It is not clear whether intrinsic defects of phagocytes of AIDS patients are responsible for their diminished anti-microbial activity. Both normal and impaired phagocytosis and intracellular killing have been reported for phagocytic cells of AIDS patients. The microbicidal activities of granulocytes and monocytes of adult AIDS patients for *Staphylococcus aureus* and Candida species are summarized in Table 1 [7–15]. Analysis of these data showed that 29 of 49 patients showed decreased phagocytosis of *C. albicans* by granulocytes; 23 of these 29 patients were parenteral drug users. It has been postulated that the impairment of granulocyte phagocytosis is related to drug abuse rather than to infection with HIV, since normal

phagocytosis of *C. albicans* by granulocytes was found in homosexual men with AIDS [8,11]. Other investigators found normal phagocytosis of *C. albicans* by granulocytes in drug abusers [9]. Monocytes did not exhibit impaired phagocytosis of *C. albicans* in the seven patients studied. Impaired phagocytosis of *C. pseudotropicalis* by granulocytes or monocytes was not observed. In four out of six patients studied, the phagocytosis of *S. aureus* by granulocytes was impaired. Killing of *C. albicans* by granulocytes was decreased in 17 out of 33 patients all except one of whom were also parenteral drug abusers. In four patients *C. pseudotropicalis* was normally killed by granulocytes, but killing by monocytes was decreased. The intracellular killing of *S. aureus* by granulocytes was reported to be diminished in 18 out of 23 patients, and by monocytes in all five patients studied. In all studies the intracellular killing was assessed during continuous phagocytosis, which makes it impossible to discern whether changes found in the intracellular killing are in fact due to a decreased rate of phagocytosis.

The aim of the present study was to study the phagocytosis and intracellular killing of *S. aureus* by granulocytes and monocytes of AIDS patients with the use of a method allowing determination of the rate of intracellular killing independently of the rate of phagocytosis, and the results are compared with those obtained with phagocytes of healthy individuals.

SUBJECTS AND METHODS

Subjects

Fifteen men with AIDS treated at the Department of Infectious Diseases of the University Hospital Leiden or at the Department of Internal Medicine of the Slotervaart Hospital in Amsterdam

Table 1. Published data on microbicidial activity of monocytes and granulocytes from adult patients with AIDS*

Number of patients	Phagocytosis of micro-organisms	Killing of micro-organisms	Reference no.
6	*C. albicans* by granulocytes decreased	*S. aureus* by granulocytes decreased	[7]
20 10 homosexual men 10 PDU	*C. albicans* by granulocytes normal decreased	*C. albicans* by granulogytes normal decreased	[8]
13 with candidiasis of which 8 PDU	*C. albicans* by granulocytes 3 patients decreased (PDU)	*C. albicans* by granulocytes 7 patients decreased (6 PDU)	[9]
6	*S. aureus* by granulocytes of 4 patients decreased	*S. aureus* by granulocytes of 1 patient decreased	[10]
10 with AIDS all PDU	*C. albicans* by granulocytes decreased	not studied	[11]
11	not studied	*S. aureus* by granulocytes decreased	[12]
7	*C. albicans* by monocytes normal	not studied	[13]
4	*C. pseudotropicalis* by monocytes normal by granulocytes normal	*C. pseudotropicalis* by monocytes decreased, by granulocytes normal	[14]
5	not studied	*S. aureus* by monocytes decreased	[15]

* Selected from publications are only the results concerning phagocytosis and intracellular killing of *S. aureus* and Candida species by granulocytes or monocytes of adult AIDS patients.
   PDA, parenteral drug user.

were included in the study. All patients were classified as belonging to group IV according to the CDC classification, and had $0.2 \times 10^9/l$ or fewer CD4+ lymphocytes; further selection on the basis of concomitant infection was not applied. The age of the patients ranged between 21 and 44 years (mean 35·3). Twelve of them had acquired the HIV via homosexual contacts, two via heterosexual contacts, and one was a parenteral drug user. Ten of the patients used zidovudine, one used dideoxyinosine, four were not taking any anti-retroviral drug. Five of the subjects were taking cotrimoxazol for secondary *Pneumocystis carinii* pneumonia prophylaxis (PCPP), and four were treated with cotrimoxazol for primary PCPP. Two patients were treated with pentamidine aerosol for secondary PCPP, one of them was taking pyrimethamine as well to prevent a relapse of Toxoplasma encephalitis. One patient was taking ganciclovir in addition to zidovudine and cotrimoxazol because of CMV retinitis, IFN-α for Kaposi's sarcoma, and fluconazole for a Candida oesophagitis. One patient used fluconazole in addition to dideoxyinosine and cotrimoxazol for Candida oesophagitis. One patient received spiramycin to suppress a persistent Cryptosporidium enteritis. Healthy donors of the blood bank of the Leiden University Hospital were used as controls.

Isolation of granulocytes and monocytes
Leucocytes from AIDS patients were isolated by Ficoll–Hypaque gradient centrifugation from blood containing sodium citrate as anti-coagulant [16]. Control cells were isolated from fresh buffy-coats. In short, blood or buffy coat was diluted with PBS (pH 7·4); 20 ml of each suspension was layered on 10 ml Ficoll–Hypaque solution (P=1·077; Pharmacia, Uppsala, Sweden) before being centrifuged at 440 *g* at 18°C for 20 min.
    Pellets containing granulocytes and erythrocyte were resuspended in PBS containing 0·5 U/ml heparin (PBS-hep). The

granulocytes were purified by dextran 1 *g* sedimentation at 37°C for 10 min using Plasmasteril (Frenius, Bad Homburg, Germany). The remaining erythrocytes were removed by a single hypotonic lysis. The cells were then washed twice with PBS-hep at 160 *g* at 18°C for 10 min and resuspended in HBSS containing 10 mM HEPES and 0·1% (w/v) gelatin (HBSS-gel) at a concentration of 1×10^7 granulocytes/ml. Cell viability, checked by trypan blue exclusion, was more than 95%.
    A suspension of 40% monocytes and 60% lymphocytes, obtained after centrifugation of the leucocytes in the Ficoll–Hypaque solution, were collected into siliconized glass tubes and washed twice with ice-cold PBS-hep by centrifugation at 160 *g* at 4°C for 10 min. Next, the cells were suspended in HBSS-gel at a concentration of 1×10^7 monocytes/ml. Cell viability amounted to more than 95%.

Micro-organism
Staphylococcus aureus (type 42D) cultured overnight in nutrient broth no. 2 (Oxoid) at 37°C were washed twice with PBS at 1500 *g* for 10 min and resuspended in HBSS-gel at a concentration of 1×10^7 bacteria/ml. Pre-opsonization of *S. aureus* was performed by incubation of 1×10^7 bacteria/ml HBSS-gel with 10% normal human serum (originating from a healthy donor with blood group AB and further to be referred to as serum) at 37°C under slow rotation (4 rev/min) for 30 min. Next, the bacteria were washed twice with cold HBSS-gel by centrifugation at 1500 *g* at 18°C for 10 min and resuspended in HBSS-gel at a concentration of 1×10^7/ml.

Phagocytosis assay
Phagocytosis of *S. aureus* by human granulocytes or monocytes was assessed as described [17,18]. Briefly, a suspension of 5×10^6 granulocytes or monocytes/ml and 5×10^7 *S. aureus*/ml HBSS-
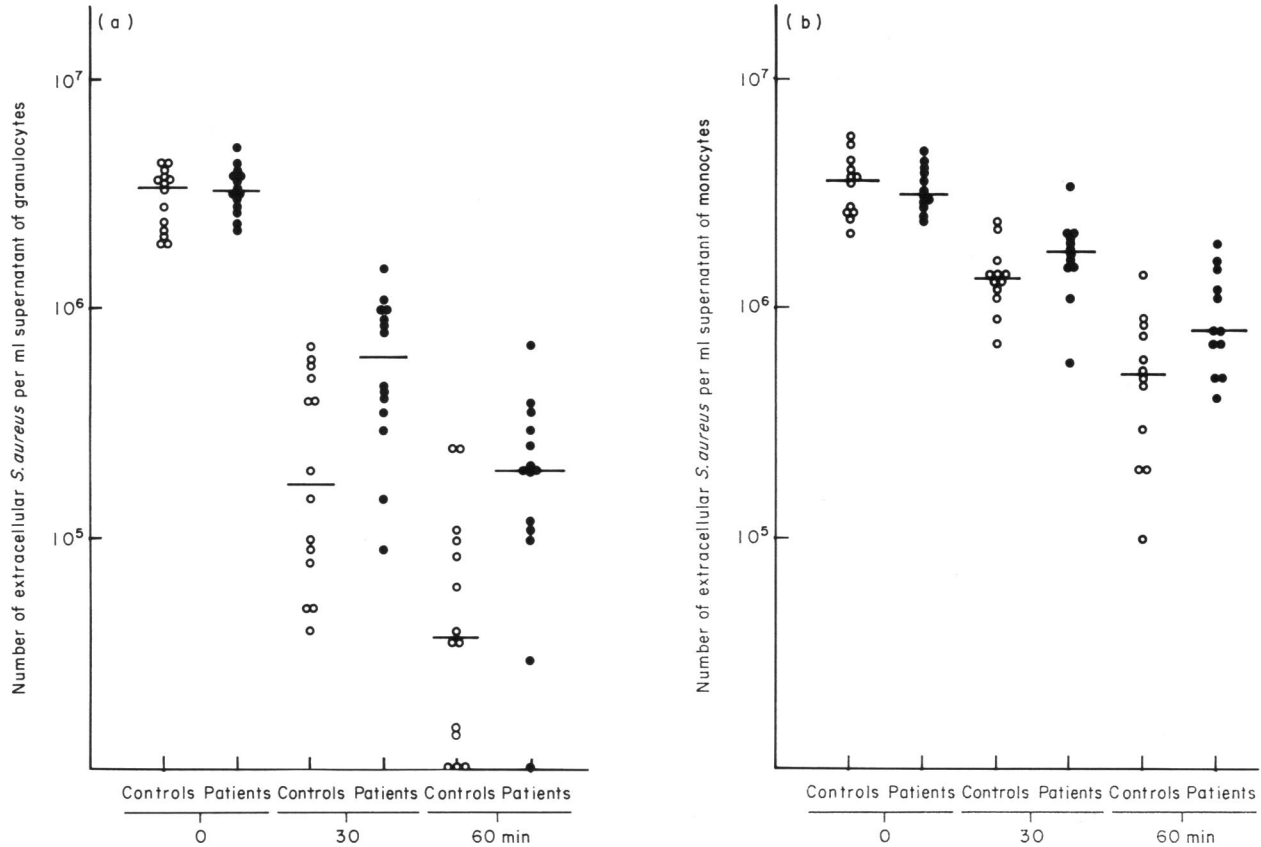
Fig. 1. Phagocytosis of *S. aureus* by granulocytes (a) and monocytes (b) of AIDS patients and controls. The decrease in the number of viable extracellular bacteria in the supernatant during incubation together with granulocytes ($n = 14$) or monocytes ($n = 12$) of patients and controls represents the phagocytosis of *S. aureus*.

Table 2. Rate constants of phagocytosis and intracellular killing of *S. aureus* by granulocytes and monocytes of AIDS patients and control*

Assay	n†	Granulocytes		n†	Monocytes	
		Controls	Patients		Controls	Patients
Phagocytosis	14	0.066 ± 0.017‡	0.050 ± 0.016‡	12	0.034 ± 0.011§	0.023 ± 0.010§
Intracellular killing	10	0.033 ± 0.016	0.030 ± 0.014	11	0.018 ± 0.011	0.012 ± 0.014

* Calculated over a 60-min assay period.
† Number of patients and controls.
‡ $P = 0.03$.
§ $P = 0.005$.

gel was incubated in the presence of 10% serum at 37°C under slow rotation (4 rev/min). At various time-points, a sample of the mixture was taken, diluted 10 times in ice-cold HBSS-gel and centrifuged at 110 g at 4°C for 4 min to separate the extracellular bacteria from the cell-associated bacteria. Ten-fold dilutions of the supernatant containing the non-ingested *S. aureus* were plated on DST-agar plates (Oxoid). After overnight incubation of the plates at 37°C, the colony-forming units (CFU) were counted. Phagocytosis is expressed as decrease in the number of extracellular *S. aureus*. The rate constant of phagocytosis (K_{ph}) during the initial 60 min was calculated according to the formula

$$K_{ph} = [\ln N_{(t=0)} - \ln N_{(t)}]/t,$$

in which $N_{(t)}$ represents the number of viable extracellular bacteria at time $= t$ and $N_{(t=0)}$ that of extracellular bacteria at the start of the phagocytosis assay.

Intracellular killing assay
Intracellular killing of *S. aureus* by human granulocytes and monocytes was assessed as described [18,19]. 5×10^6/ml granulocytes or monocytes and 5×10^6/ml preopsonized *S. aureus*

Table 3. Number of intracellular *S. aureus* in granulocytes and monocytes of AIDS patients and controls after a short period of phagocytosis*

Cells	10^6 intracellular bacteria/5×10^6 cells	
	Controls	Patients
Granulocytes	1·55±1·32†	0·69±0·48†
Monocytes	1·42±0·70‡	0·80±0·58‡

* The number of viable intracellular *S. aureus* in cells of patients and controls was determined after incubation of cells and preopsonized bacteria at a ratio of 1:1 at 37°C for 3 min.
† $P=0·03$.
‡ $P=0·05$.

were incubated at 37°C under slow rotation (4 rev/min) for 3 min to allow phagocytosis. Next, the suspension was placed on ice to terminate and prevent intracellular killing of ingested *S. aureus* and centrifuged at 110 g at 4°C for 4 min to remove extracellular, non-ingested, bacteria. The cells were then washed twice with ice-cold HBSS-gel and centrifuged at 110 g at 4°C for 4 min. The phagocytes containing ingested bacteria were resuspended at a concentration of 5×10^6/ml in HBSS-gel containing 10% serum for graunulocytes or 15% serum for monocytes, incubated at 37°C under slow rotation (4 rev/min), and at various time-points a sample was taken and diluted ten times in ice-cold water containing 0·1% (w/v) bovine serum albumin. After mixing for 1 min, the number of viable cell-associated bacteria was determined microbiologically as described above. Intracellular killing is expressed as the decrease in the number of viable cell-associated *S. aureus*. The rate constant of intracellular killing (K_k) during a certain time-period was calculated according to the formula

$$K_k = [\ln N_{(t=0)} - \ln N_{(t)}]/t,$$

in which N_t is the number of viable intracellular bacteria at time $=t$, and $N_{(t=0)}$ that of intracellular bacteria at the start of the killing assay.

Statistical analysis

Data concerning patient cells were only used when those obtained for control cells in the same experiment were comparable to published previously for control granulocytes and monocytes of healthy individuals [17–19].

Statistical analysis of the data was performed with the Wilcoxon-signed rank test.

RESULTS

Phagocytosis of S. aureus *by granulocytes and monocytes from AIDS patients and controls*

Control granulocytes showed rapid phagocytosis of *S. aureus*; after 60 min of incubation, these granulocytes had ingested 97±3% of the initial number of *S. aureus*. The granulocytes of AIDS patients phagocytosed significantly fewer *S. aureus* than control granulocytes did ($P<0·03$; Fig. 1a) and the rates

differed significantly (Table 2). Monocytes from AIDS patients ingested significantly ($P<0·005$) fewer bacteria than control monocytes did (Fig. 1b), which is also expressed in the respective rates of phagocytosis (Table 2).

Intracellular killing of S. aureus *by granulocytes and monocytes from AIDS patients and controls*

The numbers of *S. aureus* found intracellularly in granulocytes or monocytes of AIDS patients before the killing assay started were significantly lower than those in the phagocytes of controls (Table 3). The rates of intracellular killing of granulocytes and monocytes from AIDS patients and controls did not differ significantly (Fig. 2a, Fig. 2b; Table 2).

Effect of co-trimoxazole or zidovudine on the microbicidal functions of granulocytes and monocytes

To exclude the possibility that treatment of the patients with sulphamethoxazole and trimethoprim (cotrimoxazol), leading to maximal blood concentrations of 40–90 μg/ml and 2–4 μg/ml, respectively, affected the microbicidal functioning of the patient phagocytes, the effect of these drugs on the phagocytosis and intracellular killing was investigated. Granulocytes or monocytes from healthy individuals were incubated in the presence of 90 μg/ml sulphamethoxazole and 3 μg/ml trimethoprim or without these drugs for 18 h at 37°C, and then washed three times with PBS. The rates of phagocytosis and intracellular killing of *S. aureus* by cotrimoxazol-treated and control granulocytes or monocytes did not differ significantly (data not shown). Treatment of patients with zidovudine (Retrovir) leads to serum peak levels between 0·5 and 2·5 μg/ml [20]. The growth of *S. aureus* in the presence of 0·1, 1 or 10 μg/ml zidovudine was not impaired (results not shown). The rates of phagocytosis and intracellular killing of *S. aureus* by granulocytes and monocytes, that were first incubated with 1μl/ml zidovudine for 20 h and control cells were not different as well (data not shown).

DISCUSSION

From the present findings it may be concluded that monocytes and granulocytes of AIDS patients show decreased phagocytosis but normal intracellular killing of *S. aureus*. This conclusion is based on the significantly lower rates of phagocytosis by phagocytes of AIDS patients and controls and on the lower numbers of viable intracellular *S. aureus* in phagocytes of AIDS patients after 3 min of phagocytosis at the start of the killing assay.

The present study was restricted to adult AIDS patients (CDC stage IV) and healthy blood donors, and no relationship was found between the experimental results and any other clinical characteristics of the patients. A possible effect of the treatment of the patients with cotrimoxazol and zidovudine was excluded by the control experiments. If these drugs had any effect they would kill *S. aureus*, and therefore an increase in the rates of phagocytosis and intracellular killing of *S. aureus* by patient's phagocytes would be expected. This was not found.

The microbicidal functions of phagocytes of AIDS patients have been assessed in a number of other studies as well. The contradictory results of these and our studies might be explained by differences between the patients under study with respect to age, stage of disease, presence of other complicating factors, and anti-microbial treatment. The impaired intracellular killing of
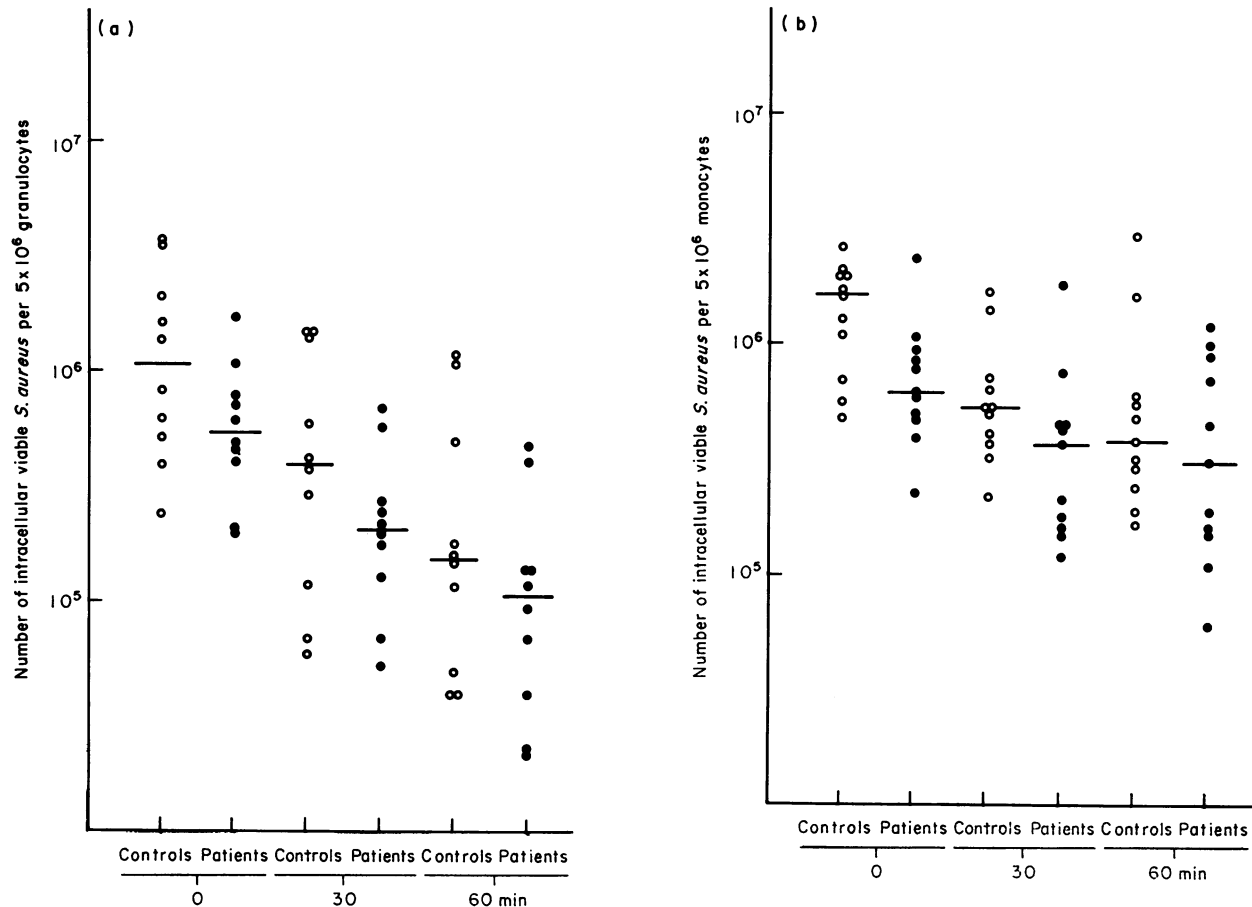
Fig. 2. Intracellular killing of *S. aureus* by granulocytes (a) and monocytes (b) of AIDS patients and controls. The decrease of the number of viable intracellular bacteria in granulocytes ($n=10$) or monocytes ($n=11$) of patients and controls represents the intracellular killing of *S. aureus*.

C. albicans by granulocytes of AIDS patients who were also parenteral drug users [8], indicate that other factors too can affect phagocyte functions. Another difference between our and other studies is that in the latter, the intracellular killing was assessed during ongoing phagocytosis, which means that a change in intracellular killing could also have been due to a change in the rate of phagocytosis. In the present study this problem was avoided, because intracellular killing was measured after 3 min of phagocytosis of opsonized bacteria, after which non-ingested *S. aureus* were removed before the intracellular killing assay was started [1].

The impaired phagocytosis of bacteria by patients' cells cannot be explained easily. Let us consider the process of phagocytosis, which involves various steps. A difference in opsonization could not have been responsible for the observed impairment, because the same serum and suspension of bacteria were used to assess the functions of patients and control cells in a simultaneous experiment. If the results for the control cells differed by more than two standard deviations from published results for healthy individuals [17–19], the experiment was discarded. A decrease in the binding of opsonized bacteria to phagocytes because of a decreased expression of Fcγ and/or complement receptors [21,22] can lead to impaired phagocytosis. Another possible explanation is that defective signal transduction induced by HIV proteins, as reported for CD4+ T

lymphocytes [23,24], accounts for the impaired phagocytosis of bacteria by patients' phagocytes, by affecting the assembly and disassembly of the actin filaments involved in the formation of pseudopodia to engulf bacteria. It is unlikely that the impaired phagocytosis of bacteria is caused by the infection of phagocytes with HIV. Monocytes infected with HIV *in vitro* display decreased antimicrobial activity [25], but a small number of the monocytes are infected with HIV *in vivo* [26]. An impaired production of cytokines, such as interferon-γ, due to the reduced number of circulating T (CD4+) lymphocytes in AIDS patients could affect the microbicidal functions of patient cells, because the latter reside in a relatively cytokine-poor environment [6]. It is not known, however, whether under normal conditions leucocytes in the circulation and tissues are affected, e.g. stimulated, by cytokines occurring under normal conditions.

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