Impaired Macrophage Phagocytosis of Apoptotic Neutrophils in Patients with Human Immunodeficiency Virus Type 1 Infection

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Dysfunction of neutrophils (polymorphonuclear leukocytes [PMNL]) and macrophagic cells occurs as a consequence of human immunodeficiency virus type 1 (HIV-1) infection. Macrophages contribute to the resolution of early inflammation ingesting PMNL apoptotic bodies. This study investigated macrophage ability to phagocytose PMNL apoptotic bodies in patients with HIV-1 infection in comparison with uninfected individuals and the effect of HIV Nef protein on apoptotic body phagocytosis to determine if phagocytic activity is impaired by HIV infection. Monocytes/macrophages were isolated from 10 HIV-1-infected patients and from five healthy volunteers, whereas PMNL were isolated from healthy volunteers. Macrophage phagocytosis of apoptotic PMNL was determined by staining of apoptotic bodies with fluorescein-conjugated concanavalin A or with fluorescein-labeled phalloidin. Our data show significant impairment of PMNL apoptotic body macrophage phagocytosis in subjects with HIV-1 infection presenting a concentration of CD4+ T lymphocytes of >200/mm³ and in particular in those with <200 CD4+ T lymphocyte cells/mm³. In addition, HIV-1 recombinant Nef protein is able to decrease phagocytosis of apoptotic PMNL from normal human macrophages in a dose-dependent manner. The results of our study suggest that impaired macrophage phagocytosis of PMNL apoptotic bodies may contribute to the persistence of the inflammatory state in HIV-infected subjects, especially during opportunistic infections that are often favored by defective phagocytic activity.

Neutrophil (polymorphonuclear leukocytes [PMNL]) function, including chemotaxis, phagocytosis, oxidative burst capacity, and bacterial killing, is impaired in the course of human immunodeficiency virus type 1 (HIV-1) infection, particularly in the later stages of the disease, and this abnormal function may predispose to some secondary bacterial infections and/or to opportunistic infections (4, 8, 10, 11, 12). PMNL have the shortest half-life of all circulating leukocytes and are programmed to die within 1 day. These aging leukocytes spontaneously undergo apoptosis and are recognized and phagocytosed by macrophages (15).

Pitark et al. (13) have demonstrated that the rate of PMNL apoptosis is accelerated in AIDS patients, and this defect is intrinsic and not an effect of endogenous serum factors. Moreover, it has been proposed that the engulfment of apoptotic PMNL triggers production of anti-inflammatory mediators from macrophages (6, 9), whereas persistent PMNL-rich inflammatory infiltrates have been associated with unresolved inflammatory reactions, including adult respiratory distress syndrome and rheumatoid arthritis (16). Thus, the removal of apoptotic cells appears to be critical in the resolution of inflammation. We have previously demonstrated that macrophages from HIV-positive subjects have a reduced ability to phagocytose Candida albicans cells, and there is a significant decrease in oxidative processes for the intracellular killing. These phenomena seem to be induced, at least in part, by HIV Nef protein (14).

Since the effects of macrophage phagocytosis of apoptotic PMNL have not been completely investigated, especially in HIV-positive subjects, the purpose of this study was to evaluate phagocytosis of PMNL apoptotic bodies performed by macrophagic cells obtained from HIV-1-positive subjects and in parallel by the macrophages obtained from healthy individuals. Furthermore, we studied the effect of Nef protein on PMNL apoptotic body macrophagic phagocytosis, since this viral protein is able to depress both specific and nonspecific immune responses in HIV-infected patients, particularly microbial phagocytosis (1, 14).

MATERIALS AND METHODS

Subjects. Ten HIV-1-infected subjects (mean age, 35.3 ± 5.8 years) were enrolled and five healthy volunteers (mean age, 37.1 ± 4.4 years), without HIV-1 risk factors, served as controls. Five of the HIV-1-infected subjects presented more than 200 CD4+ T lymphocytes/mm³ (mean = 517 ± 225), had a CD4/CD8 T-cell ratio of 0.5 ± 0.1, and had mean HIV-1 RNA levels in plasma of 32,628 ± 42,188 copies/ml. Five patients had less than 200 CD4+ T lymphocytes/mm³ (mean = 101 ± 70), a ratio of 0.2 ± 0.2, and mean HIV-1 RNA levels of 375,000 ± 246,815/ml. All these patients had a moderate anemic state and were generally studied before receiving antiretroviral therapy. In fact, a significant part of our study population included individuals who had ignored their seropositive condition for a long time and in consequence came to medical evaluation late.

Monocyte and PMNL isolation and apoptotic body preparation. Monocytes obtained from peripheral blood of healthy subjects and HIV-1-infected patients were selected as adhering cells after separation of peripheral blood mononuclear cells with a Ficoll Paque gradient (Pharmacia, Uppsala, Sweden). After repeated
were washed twice by centrifugation for 10 min at 3,000 rpm and resuspended in (14). For this purpose we used the antioxidant power (PAO) kit furnished by oxidative phenomena of macrophages in the absence or presence of Nef protein, by‘sation. The differences among the experimental and control groups were statisti-
sponding to PAO units.
fonate), and it was measured spectrophotometrically at 490 nm (Metertech/H11001
Baltimore, Md.), the cells were harvested by trypsin treatment and transferred,
phage colony-stimulating factor (10 ng/ml; Genzyme, Milan, Italy) was added to
lymphocytes (23.9% ± 10%).
low up the assay. Phagocytosed apoptotic bodies were resuspended at the indi-
cal concentration of 5 × 10^6/ml to obtain a ratio of 5:1 per macrophagic cell (this preparation was diluted 1:2 in the wells containing macrophage cells). This method allows uniform staining of the apo-
totic body surface, making the apoptotic bodies easily detectable in macroph-
ag ic cells without having any significant influence on phagocytosing activit y.
In the second method, fluorescein-labeled phalloidin (Sigma-Aldrich Chemic-
cals, Milan, Italy) was used at a final concentration of 5 μg/ml. This product is
able to stain the interior of the apoptotic bodies, binding firmly to the microtu-
bular structures. This staining method was used like the first one.
In a subsequent set of experiments, macrophage phagocytosis of PMNL apo-
totic bodies was also investigated in the presence of various concentrations of recombinant Nef protein (American Biotechnologies, Inc., Cambridge, Mass.),
using macrophagic cells obtained from four healthy individuals and performing
the experiments in triplicate. The challenge with stained apoptotic bodies, using
both the first and second methods, was made at a ratio of 5:1.
After 2 h of incubation in the presence of apoptotic bodies, the supernatants
were removed, and slides were detached from their supports and washed twice
with phosphate-buffered saline (PBS). Then the slides were fixed with 10% ethyl
alcohol solution, mounted in phosphate-buffered glycerol (30% PBS and 70%
 glycerol, vol/vol), and examined under a fluorescent microscope at ×400 by
cfour blinded microscopists. At least five microscopic fields were observed
for each sample.
Macrophage oxidative phenomenon evaluation. We indirectly studied the ox-
idative phenomena of macrophages in the absence or presence of Nef protein, by
evaluating the antioxidant power of the supernatants, as previously described
(14). For this purpose we used the antioxidant power (PAO) kit furnished by
Med. Dia S.r.l. (San Geramo, Vercelli, Italy). The test is based on the detection of Cu^+ ions produced by the reduction of a known amount of Cu^{2+}; this reduction is induced by the antioxidant factors present in the culture, the activity of which decreases in an inversely proportional manner to the activity of mac-
rophage oxidative phenomena. The concentration of Cu^{2+} ion was detected
through the formation of complexes consisting of Cu^2+ and the chelating agent
betacaptoprime disulfonate (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroinedisul-
fonate), and it was measured spectrophotometrically at 490 nm (Metertech
spectrophotometer; Medical System, Genoa, Italy). The data are expressed as
microequivalents per liter of reducing equivalents ± standard deviation, corre-
sponding to PAO units.
Statistical analysis. The results were expressed as mean and standard devia-
tion. The differences among the experimental and control groups were statisti-
cally evaluated using Student’s t test. Statistical significance was defined as P ≤
0.05. Moreover, a linear correlation test was employed for the correlation study
reported in the results.

**RESULTS**

In the first set of experiments, we studied the phagocytosis of PMNL apoptotic bodies stained with fluoresceinated ConA from macrophages of patients with HIV-1 infection and from those of uninfected healthy individuals.

A significant decrease in the percentage of phagocytosing cells was observed in HIV-1-positive subjects presenting a CD4^+ T-cell concentration of >200 cells/mm^3 (24.2% ± 12.5%; P = 0.002) and in those with <200 CD4^+ T-cells/mm^3 (23.7% ± 6.9%; P < 0.001), compared to healthy control subjects (50.1% ± 3.7%) (Table 1). We point out that by analyzing all the HIV-positive subjects, the mean number of phagocytosing cells was 23.9% ± 10% in 52% of the controls (P < 0.001). Since fluoresceinated ConA only stains the surface of apoptotic bodies, we performed further experiments with fluoresceinated phalloidin, which is endowed with a specific ability to bind to microtubular structures. We also observed a reduction of about 50% in phagocytosing cells in HIV-positive subjects compared to the controls (unreported data).

As reported in Table 2, a significant decrease in the number of PMNL apoptotic bodies phagocytosed per macrophage cell was observed in HIV-1 subjects with >200 CD4 T-cells/mm^3 (0.6 ± 0.4; P = 0.001) and in those with <200 CD4^+ T-cells/mm^3 (0.3 ± 0.1; P < 0.001) compared to the controls (2.4 ± 0.7).

Evaluating all the HIV-positive subjects, the mean number of apoptotic bodies phagocytosed per cell was 0.45 ± 0.3 (P < 0.001). As can be seen in Table 2, the reduction of the mean number of PMNL apoptotic bodies phagocytosed per cell in HIV-positive subjects compared to the controls is fivefold, and the percent reduction in phagocytosing cells in infected patients is twofold. This observation suggests a decreased ability of single phagocytosing cells to swallow apoptotic bodies.

In a further set of experiments, we studied the phagocytic activity of PMNL apoptotic bodies in normal human macro-

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**TABLE 2. Number of PMNL apoptotic bodies phagocytosed per macrophage**

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Mean no. of apoptotic PMNL/macrophage ± SD</th>
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<tbody>
<tr>
<td>Controls</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>HIV-positive subjects with &gt;200 CD4^+ T-cells/μl</td>
<td>0.6 ± 0.4*</td>
</tr>
<tr>
<td>HIV-positive subjects with &lt;200 CD4^+ T-cells/μl</td>
<td>0.3 ± 0.1**</td>
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</tbody>
</table>

* *, P = 0.001 compared with controls; ***, P < 0.001 compared with controls; P < 0.001 for controls versus all HIV-seropositive subjects (0.45% ± 0.3%).
particles (14). The decrease in oxidative processes and the inhibition of macrophagic function seem to occur in parallel.

Aging PMNL spontaneously undergo apoptosis and are recognized and phagocytosed by monocytes and macrophages (15). PMNL function is impaired in all stages of HIV-1 infection and especially in the terminal stage of the disease (17). Pitrak et al. (13) have shown that abnormalities of PMNL function observed in HIV-positive subjects might partly depend on the accelerated apoptosis induced by HIV infection. In fact, during all stages of HIV-1 infection, there is an increased number of apoptotic PMNL which are unable to function as host defenders (2, 17). The removal of apoptotic cells appears to be central to the resolution of inflammation. In fact, the clearance of apoptotic PMNL not only prevents the release of toxic and immunogenic intracellular contents, but also stimulates the macrophages to produce inflammatory mediators, including transforming growth factor β1, prostaglandin E₂, and platelet-activating factor, and inhibits the production of tumor necrosis factor, interleukin-1β, and interleukin-8 (6).

Dysregulation of PMNL function, along with that of monocytic macrophagic cells, in HIV-1-infected patients is reflected in the increased incidence of some microbial infections among these patients (5, 7). During several microbial infections, PMNL migrate and accumulate at the inflammatory sites, followed by removal of inflammatory cells. This occurs mainly by apoptosis and by phagocytosis of apoptotic bodies. In parallel, some antimicrobial pathogens can be phagocytosed and killed by macrophages. These phenomena appears to be critical to the resolution of inflammation and infection. However, the decreased phagocytosis of apoptotic PMNL by macrophages in HIV-1-infected patients and the accelerated apoptosis of PMNL lead to accumulation of apoptotic inflammatory PMNL and a decrease in their clearance. The persistence of apoptotic PMNL and their apoptotic bodies at the inflammatory site may maintain the inflammatory state through persistent stimulation of proinflammatory cytokines (6). This can explain some of the pathological conditions in the gastrointestinal tract or bronchoalveolar tract that have been reported (3).

**REFERENCES**