# Phagocytosis of Nanoparticles by Human Immunodeficiency Virus (HIV)-Infected Macrophages: A Possibility for Antiviral Drug Targeting

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Human monocytes/macrophages (MO/MAC) were isolated from peripheral blood and cultivated on hydrophobic Teflon membranes. This culture system is suitable for HIV infection of MO/MAC *in vitro*. After transfer into 24-well plates the mature macrophages (infected or uninfected) were used for measurements of phagocytosis. The uptake of different, radioactively labeled nanoparticles (NP) made of polyalkylcyanoacrylate, polymethylmethacrylate (PMMA), and human serum albumin (HSA) by the macrophages was determined. In addition, the influence on phagocytosis of size and composition, concentration, and surface of the NP was studied. Further, macrophages of different state of activation were tested. NP made of polyhexylcyanoacrylate (PHCA) or human serum albumin with a diameter of about 200 nm were found most useful for targeting antiviral substances such as azidotymidine to macrophages. Cells infected *in vitro* with HIV-$1_{D117/III}$, a monocytotropic HIV isolate from a perinatally infected child, possessed an even higher phagocytotic activity than noninfected cells. Macrophages isolated from HIV-infected patients also showed good incorporation of NP. Thus, the concept of a specific targeting of antiviral substances to macrophages in HIV-infected individuals appears quite promising.

KEY WORDS: nanoparticles; human immunodeficiency virus (HIV); macrophage; drug targeting; phagocytosis.

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[6] Abbreviations used: MO/MAC, monocytes/macrophages; PBMC, peripheral blood mononuclear cells; MPS, mononuclear phagocyte system; IFN, interferon; NP, nanoparticle(s); PBCA, polybutylcyanoacrylate; PHCA, polyhexylcyanoacrylate; PMMA, polymethylmethacrylate; HSA, human serum albumin; HIV, human immunodeficiency virus; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

## INTRODUCTION

The mononuclear phagocyte system (MPS)[6] comprises cells of considerable heterogeneity in terms of localization, metabolism, and function (1). Originating from early hemopoietic progenitor cells in the bone marrow, circulating blood monocytes (MO) undergo further maturation into macrophages (MAC) upon migration from the capillary bed to tissue sites of inflammation (2). *In vitro*, MO-to-MAC maturation can be followed by the expression of specific maturation-associated antigens on the cell surface (3) and is accompanied by a characteristic change in the secretory repertoire of MAC in comparison to MO (4).

Apart from the CD4 T-helper subset of lymphocytes, cells of the MPS can be infected by HIV-1 and HIV-2 strains (5–7). For monocytes/macrophages (MO/MAC) the virus seems to be much less cytocidal than for T cells. Therefore, cells of the monocyte/macrophage lineage most likely play an important role in the immunopathogenesis of the HIV infection, by serving as a reservoir for the virus and its dissemination throughout the body and brain (8,9).

Nanoparticles (NP) are stable, spherical particles between 100 and 1000 nm in size, with a continuous matrix. NP vary in materials, size, and surface properties. Administered intravenously, they very rapidly distribute among the tissues of the MPS. Due to phagocytosis by monocytes/macrophages, the NP enrich in liver, spleen, bone marrow, lymph nodes, and blood (10,11). Therefore, using NP in order to target drugs to these tissues is an attractive concept (12). In nuclear medicine, NP have already been used for scintigraphy of bone marrow and other organs (13,14). The distribution of the NP can further be influenced by manipulations such as coating of the surface with specific target molecules or with the aim of changing the hydrophilicity/lipophilicity. This additional targeting effect was demonstrated successfully in mice and rat (15,16).

Because of the properties mentioned above, NP payloaded with antiviral drugs should be suitable for targeting these substances to uninfected and HIV-infected MO/MAC, thus allowing reduction of the required dose and minimization of the toxicity of such drugs.

In this study, the particle material and size of the NP were optimized for drug targeting using human macrophages which derived from *in vitro* differentiated bloodborne monocytes. Additionally, possibilities to improve the phagocytosis of the NP by variation of concentration, surface, and other parameters were investigated. Finally, phagocytosis of the NP was examined either by MO/MAC infected with HIV *in vitro* or by MAC isolated from HIV-infected patients from different stages of disease.

## MATERIALS AND METHODS

*Isolation and Culture of Monocytes/Macrophages (MO/MAC)*. Fresh human mononuclear cells (PBMC) from healthy blood donors were prepared by separation on a Ficoll gradient and cultivated in supplemented RPMI 1640 medium (Biochrom, FRG) with 5% human AB serum on hydrophobic Teflon membranes (17). In the same manner



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isolation of MO/MAC from HIV-infected patients was performed. The patients in this study are participants of the "Frankfurter HIV-Model." They were classified according to the CDC recommendations (18,19). Three days after the start of the culture, the cells were transferred to 24-well plates, where the nonadherent cells were quantitatively washed off. The adherent cells ($1–2 \times 10^5$ monocytes/macrophages per well) were further cultivated in 2 ml supplemented RPMI 1640 medium with 5% human AB serum. According to the morphology and expression of CD14 antigen, the remaining cell layer consisted of up to 95% macrophages.

*Infection of MO/MAC with HIV-1.* On day 1 from the start of the culture, the PBMC were infected in the Teflon bags with 1 ml stock virus per 10 ml cell suspension (7). On day 3, the PBMC were plated out and the virus inoculum as well as the lymphocytes was removed by washing off the nonadherent cells. The stock virus consisted of peripheral blood lymphocytes ($1 \times 10^6$/ml) infected with HIV-$1_{D117/III}$. Originally, this virus strain was isolated from a perinatally infected child. HIV-$1_{D117/III}$ is a monocytotropic strain, which replicates to high titers in MO/MAC (20). The infection of the mature MAC was proved by using an HIV antigen ELISA (Organon Teknika, FRG) or by reverse transcriptase (RT) assay (21).

*Synthesis and Purification of the Nanoparticles.* Polyalkylcyanoacrylates and polymethylmethacrylate NP were produced as described earlier (22) after labeling with $^{14}C$ by Amersham Int. (UK). NP made from human serum albumin (SIGMA, FRG) were synthesized (23,24) and labeled with $^{35}S$ (SLR-reagent, Amersham Int., UK). Purification was carried out using column chromatography (Sephadex G 50). The size of the NP was measured with a photon correlation spectrometer BI 90 (Brookhaven Inst.). Coating of the NP was carried out by suspending the freeze-dried particles in phosphate buffer containing 1% of the pluronics (16) and stirring (500 U/min) for 48 hr. Then the NP were centrifuged at 45000 U/min for 2 hr with a Ti-70 rotor.

*Phagocytosis of the Radioactively Labeled Nanoparticles.* At day 7 and day 21 from the start of the culture, the amount of phagocytosis was examined by adding the NP in supplemented medium to the cultures. The cells were incubated at 37°C for different times (0 to 24 hr). The excess of NP was removed by pipetting and the cells were washed twice with PBS to remove adherent NP from their surface. Parallel cultures at 2°C served as negative control, because at this temperature no phagocytosis can take place, as it is an energy-dependent process (25). The viability of the cells was examined by a dye exclusion test with trypan blue. Other cultures were stimulated with 200 U/ml interferon-gamma (Thomae, Biberach/Riss, FRG) for 24 hr before adding the NP. For the quantitative determination of incorporated NP, the cells were dissolved in lauroylsarcosine buffer and the radioactivity was measured with a scintillation counter.

*Preparation for Scanning Electron Microscopy.* The cells were fixed with 2.5% glutaraldehyde in cacodylate buffer (0.075 *M* cacodylic acid sodium salt, 4.5% sucrose, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$). They were postfixed with 1% $OsO_4$ before being dried with hexamethyldisilazane. The samples were then coated with gold-palladium and observed in a Hitachi S-800 electron microscope.

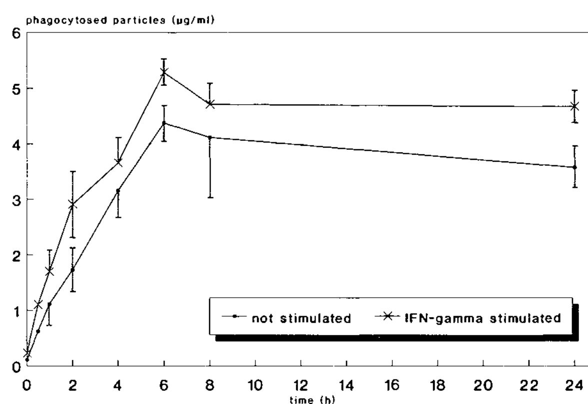


Fig. 1. Phagocytosis of NP by human MAC. NP made of HSA (200-nm diameter) were added at day 21 after start of culture at a final concentration of 0.5 mg/ml for different times (between 0 and 24 hr). The data are based on one representative experiment out of five. Mean values of three parallel cultures are presented. Bars indicate standard deviation.

## RESULTS

### Phagocytosis as a Function of Time and State of Activation

As shown in Fig. 1, phagocytosis of NP consisting of human serum albumin is terminated after 4–6 hr. Further incubation does not increase the amount of uptake, but a saturation is reached. A slightly higher amount of incorporation is achieved by stimulation of the macrophages with interferon-gamma before addition of the NP (Fig. 1). Phagocytosis and its slight stimulation by IFN are similar for all tested NP. The augmentation of phagocytosis by IFN-gamma stimulation amounts to 14–45% in the final level as determined in five independent experiments (data not shown).

### Alteration of the Surface of the NP

The uptake of the particles can be influenced by alteration of the surface of the NP. Therefore, polybutylcyanoacrylate (PBCA)-NP coated with two different, biocompatible poloxameres: Pluronic F68 and Pluronic F108 were tested. The particles (200-nm diameter) were added at day 7 at a final concentration of 0.75 mg/ml. The uptake was measured after 6 hr of incubation. Coating with the more lipophilic Pluronic F68 improved phagocytosis by nearly 50%,

Table I. Influence of Surfactant on Phagocytosis of PBCA-NP by Human MAC

| | Phagocytosed NP (µg/ml ± SD)[a] | | |
|---|---|---|---|
| | Uncoated NP | Pluronic F108 | Pluronic F68 |
| IFN[b] | 8.61 ± 1.96 | 9.45 ± 1.76 | 13.00 ± 2.91 |
| Control | 6.37 ± 0.31 | 7.05 ± 1.41 | 9.77 ± 2.55 |

[a] Mean values of three parallel cultures are presented.
[b] The cells were stimulated with IFN-gamma for 24 hr (200 U/ml).

Table II. Influence of Particle Concentration on Phagocytosis of PBCA-NP by Human MAC

| NP conc. (μg/ml) | 125 | 250 | 500 | 750 | 1000 |
|---|---|---|---|---|---|
| Phagocytosed NP (μg/ml ± SD)[a] | 1.39 ± 0.87 | 3.48 ± 1.07 | 7.39 ± 0.30 | 9.61 ± 1.96 | 24.1 ± 1.43 |

[a] Mean values of three parallel cultures are presented.

whereas Pluronic F108 seemed to have no influence (Table I).

### Dependence on the NP Concentration

The amount of phagocytosis depends very much on the concentration of the added NP. PBCA-NP (200-nm diameter) at concentrations between 125 and 1000 μg/ml were tested. The NP were added at day 7 to IFN-gamma-stimulated MAC (200 U/ml for 24 hr) and incubated for 6 hr. The data are shown in Table II. Particle uptake increased with increasing concentrations. At the highest concentration of 1000 μg/ml, a particle uptake of 24.1 μg NP by 1 ml of the cell suspension consisting of $1 \times 10^5$ macrophages is obtained. This amounts to an incorporation of 241 pg NP by a single cell. Considering the radius of 125 nm and the density of approximately 1.13 g/cm$^3$ of the PBCA-NP, it can be calculated that 1 pg of NP consists of 108 single particles. The uptake of one macrophage, therefore, can be approximated to about $2.6 \times 10^4$ NP. Considering that this number of particles occupies a volume of 400 μm$^3$ and that a mature MAC (with an average diameter of 30 μm) has the volume of nearly $15 \times 10^3$ μm$^3$, 2–3% of the volume of one MAC is filled with phagocytosed NP at this concentration.

### Dependence of Phagocytosis on Particle Composition and Size

The phagocytosis also depends on composition and size of the NP. NP made of different materials and with different diameters were added at day 21 to the nonstimulated cultures at a final concentration of 0.5 mg/ml and incubated for 24 hr. In agreement with the results with coated polybutylcyanoacrylate (PBCA)-NP (see Table I), the more lipophilic NP e.g. polymethylmethacrylate (PMMA) NP, were phagocytosed better (Table III) than polyalkylcyanoacrylate NP (PBCA and PHCA) of a similar size (ca. 200 nm). NP of the same material were phagocytosed to a larger extent if they were larger in diameter, e.g., the incorporation of NP made from human serum albumin (HSA) 1.5 μg in diameter is higher than of HSA-NP only 200 nm in diameter (Table III). For the HSA-NP with the larger diameter, the process of phagocytosis was demonstrated by scanning electron microscopy (Fig. 2). In the uptake of the different polyalkylcyanoacrylate NP (PBCA and PHCA), only slight differences were found.

### Influence of HIV Infection on Phagocytosis

As HIV infection is known to change functional properties of MO/MAC (26), the influence of HIV infection on the phagocytosis of the NP was examined. MO/MAC were infected with the monocytotropic strain HIV-$1_{D117/III}$ (20) and PBCA-NP were added at a concentration of 500 μg/ml at days 7 and 21 after the start of the culture. A particle uptake increased by 30% was observed at day 7 for the infected cells (Fig. 3; $P < 0.05$ according to Student's $t$ test). The difference between infected and uninfected macrophages increased to nearly 60% at day 21 (Fig. 3; $P < 0.01$). At this date, the replication rate of the virus reached its maximum (RT activity: $8 \times 10^5$ cpm/ml).

In order to investigate the phagocytotic activity of macrophages from HIV-infected patients (Table IV), the cells from seven patients classified in stage CDC II/III and seven patients in stage CDC IV were compared with cells from HIV-negative healthy blood donors. Between cells from healthy donors and cells from patients at stage CDC II/III a slight, but significant reduction in phagocytotic activity was observed ($P < 0.05$), whereas between cells from CDC IV patients and cells from healthy donors no significant difference could be found ($P > 0.5$). Cells from symptomatic patients (CDC IV) showed good phagocytosis in general and, in three cases (Nos. 14, 15, 17), even slightly elevated values. However, in two cases (Nos. 12, 13) a decreased uptake was observed. No correlation between CD4-cell counts as a parameter for immune dysfunction and the phagocytotic capacity of MAC was found.

## DISCUSSION

The results show that NP were phagocytosed by human MAC *in vitro*. The extent of phagocytosis depended on various factors. Besides activation of the cells with IFN-gamma and surface coating of the particles, the most important factors determining phagocytosis were the size and the compo-

Table III. Influence of Particle Size and Composition on Phagocytosis of NP by MAC

| Particle material | PHCA | PBCA | PMMA | HSA | HSA |
|---|---|---|---|---|---|
| Particle size (nm) | 200 | 200 | 130 | 200 | 1500 |
| Phagocytosed NP (μg/ml ± SD)[a] | 3.01 ± 0.45 | 6.56 ± 0.41 | 19.28 ± 2.82 | 4.38 ± 0.29 | 15.84 ± 2.79 |

[a] Mean values of three parallel cultures are presented.

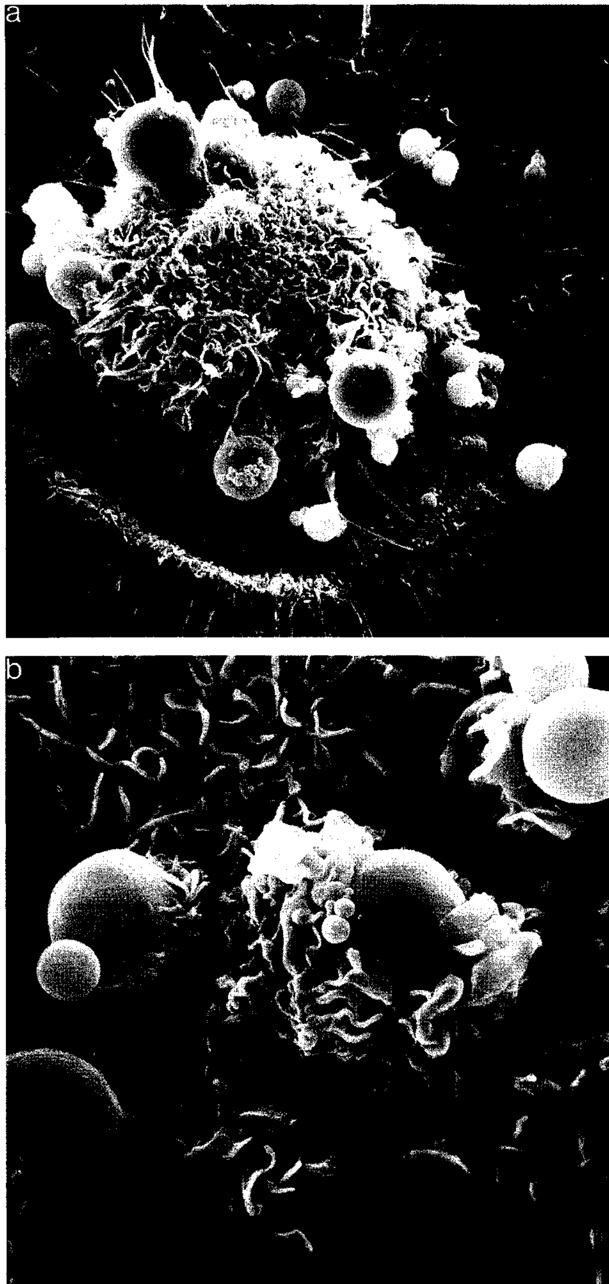


Fig. 2. Process of phagocytosis of nanoparticles (NP). (a) Numerous NP made of human serum albumin (approx. 1.5-μm diameter) attached to the surface of an HIV-infected MAC at day 20 after 30 min of incubation with the particles. (b) Attachment of NP to pseudopods (left) and engulfment by lamellipods (right). Scanning electron microscopy; (a) ×2000, (b) ×5000.

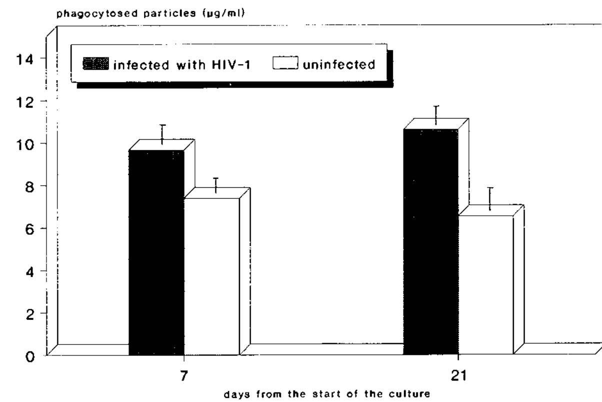


Fig. 3. Influence of HIV infection on phagocytosis of NP by human MAC. MO/MAC were infected with HIV-$1_{117/III}$ at day 1 after start of culture. At day 7 or day 21 NP made of PBCA (200-nm diameter) were added at a final concentration of 0.5 mg/ml to the infected cultures and incubated for 6 hr. The data are based on one representative experiment out of three. Mean values of three parallel cultures are presented. Bars indicate standard deviation.

sition of the NP as well as their concentration in the culture medium.

Although NP made of lipophilic materials such as PMMA and with larger diameters were more easily phagocytosed, NP consisting of HSA and polyhexylcyanoacrylate (PHCA) with a diameter of 200 nm will be used for future experiments. PMMA is only poorly degradable in the organism and therefore not applicable to our purpose. HSA-NP with a mean diameter of 1.5 μm presently are not sufficiently homogeneous for i.v. administration, because a possible agglomeration of the NP could lead to embolisms. For polyalkylcyanoacrylate it was shown that the toxicity depends on the alcohol: the toxicity decreases with increasing chain length (27) of the alcohol. Polyhexylcyanoacrylate (PHCA) is, therefore, less toxic than polybutylcyanoacrylate (PBCA). NP made of HSA show no toxic effects at all, and even at very high concentrations (>1 mg/ml) the cells were not damaged.

The most important finding of this study, however, is the observation that HIV-infected macrophages show a higher phagocytotic uptake of NP than noninfected MAC. This higher amount of phagocytosis is probably due to an activated state of these infected cells (26) and may allow a preferential phagocytosis of drug-loaded NP, resulting in a targeted delivery of drugs to these cells. In addition, our finding of good phagocytotic activity of MAC from HIV-infected patients argues that successful drug targeting should be possible. However, in two of seven AIDS patients, lower phagocytotic activity was observed, while in three of seven a higher activity was found, which was compatible with the enhanced activity seen for MAC infected with HIV *in vitro*. Further studies are needed to show whether AIDS patients fall into two different groups with enhanced or reduced phagocytotic activity.

In summary, nanoparticles hold promise for targeting antiviral substances to infected cells of the MPS. Using this technology it should be possible to reduce the administered dose of these drugs and thus to reduce their side effects.

Table IV. Phagocytotic Activity of Macrophages Isolated from HIV-Infected Patients

| Patient No. | State | CD4-positive cells/μl | CD8-positive cells/μl | Phagocytosis of PBCA particles by MAC (μg/ml ± SD)[a] |
|---|---|---|---|---|
| 1 | Uninfected control | ND | ND | 8.42 ± 0.86 |
| 2 | | ND | ND | 7.91 ± 0.70 |
| 3 | | ND | ND | 7.84 ± 0.44 |
| 4 | | ND | ND | 9.98 ± 0.51 |
| 5 | CDC/II/III | 505 | 630 | 7.02 ± 1.02 |
| 6 | | 472 | 1935 | 6.25 ± 0.97 |
| 7 | | 362 | 1729 | 5.87 ± 0.72 |
| 8 | | 627 | 493 | 5.86 ± 1.88 |
| 9 | | 669 | 698 | 4.29 ± 0.15 |
| 10 | | 709 | 902 | 3.75 ± 0.15 |
| 11 | | 672 | 797 | 5.62 ± 0.33 |
| 12 | CDC IV | 504 | 875 | 2.12 ± 0.34 |
| 13 | | 36 | 1022 | 1.71 ± 0.20 |
| 14 | | 44 | 453 | 11.22 ± 0.42 |
| 15 | | 343 | 1090 | 12.81 ± 1.61 |
| 16 | | 191 | 722 | 8.10 ± 0.75 |
| 17 | | 74 | 699 | 13.08 ± 1.33 |
| 18 | | 56 | 774 | 10.30 ± 0.83 |

[a] SD, standard deviation of three independent cultures from each patient. The data of the three patient groups were statistically compared using a *t* test (see text).

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