Available online at www.sciencedirect.com
SCIENCE DIRECT
Vaccine 22 (2004) 2910–2924
Vaccine
www.elsevier.com/locate/vaccine

Novel biocompatible anionic polymeric microspheres for the delivery of the HIV-1 Tat protein for vaccine application

Antonella Caputo [a,*], Egidio Brocca-Cofano [a], Arianna Castaldello [a], Rita De Michele [a], Giuseppe Altavilla [b], Marco Marchisio [c], Riccardo Gavioli [d], Ulrika Rolen [d], Laura Chiarantini [e], Aurora Cerasi [e], Sabrina Dominici [e], Mauro Magnani [e], Aurelio Cafaro [f], Katia Sparnacci [g], Michele Laus [g], Luisa Tondelli [h], Barbara Ensoli [f]

[a] *Department of Experimental and Diagnostic Medicine, Section of Microbiology, University of Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy*
[b] *Institute of Pathologic Anatomy and Histology, University of Padova, 35100 Padova, Italy*
[c] *Department of Morphology and Embryology, University of Ferrara, 44100 Ferrara, Italy*
[d] *Department of Biochemistry and Molecular Biology, University of Ferrara, 44100 Ferrara, Italy*
[e] *Department of Biochemistry, University of Urbino, 61029 Urbino, Italy*
[f] *Laboratory of Virology, Istituto Superiore di Sanità, 00161 Rome, Italy*
[g] *Department of Advanced Sciences and Technologies, University of Piemonte Orientale and INSTM, UdR, 15100 Alessandria, Italy*
[h] *Consiglio Nazionale delle Ricerche, I.S.O.F., 40129 Bologna, Italy*

Received 4 July 2003; accepted 2 December 2003

Available online 1 February 2004

Abstract

Two novel classes of biocompatible core-shell anionic microspheres, composed of an inner hard insoluble core, either made of poly(styrene) (PS) or poly(methyl methacrylate) (PMMA), and a soft outer tentacular shell made of long soluble negatively charged arms derived from the steric stabilizer, hemisuccinated poly(vinyl alcohol) or Eudragit L100/55, respectively, were prepared by dispersion polymerization and characterized. Five types of these novel microspheres, two made of poly(styrene) and hemisuccinated poly(vinyl alcohol) (A4 and A7), and three made of poly(methyl methacrylate) and Eudragit L100/55 (1D, 1E, H1D), differing for chemical composition, size, and surface charge density were analyzed for the delivery of the HIV-1 Tat protein for vaccine applications. All microspheres reversibly adsorbed the native biologically active HIV-1 Tat protein preventing Tat from oxidation and maintaining its biological activity, therefore increasing the shelf-life of the Tat protein vaccine. The microspheres efficiently delivered Tat intracellularly, and were not toxic in vitro nor in mice, even after multiple administrations. These results indicate that these novel microparticles are safe and represent a promising delivery system for vaccination with Tat, as well as for other subunit vaccines, particularly when a native protein conformation is required.

Keywords: Biocompatible microspheres; HIV-1 Tat protein; Vaccine

1. Introduction

The development of new adjuvants or delivery systems for protein immunization is an expanding research field [1–10]. However, a serious limitation to the use of several new adjuvants in humans is represented by their reactogenicity [11,12]. In recent years, polymeric microspheres containing protein antigens have been investigated as potential delivery systems for their capability to efficiently target the antigen to professional antigen-presenting cells and to release it in a controlled way over a prolonged period of time [1,13,14]. The use of such microparticulate protein vaccines allows to reduce the dose of antigen for primary immunization or to develop single dose vaccines, with antibody levels and cellular immune responses similar to or greater than those observed with adjuvants such as alum [2,4,9]. Successful incorporation of proteins in poly(DL-lactide) (PLA) and poly(DL-lactide-co-glicolide) (PLGA) biodegradable microparticles with respect to loading and encapsulation efficiency, as well as microparticle size and morphology, has been described in several studies [15–17]. However, although proteins encapsulated into a PLA or PLGA matrix may be protected from unfavorable conditions (e.g. pH, bile salts and proteolytic enzymes) encountered after parenteral

* Corresponding author. Tel.: +39-0532-291330; fax: +39-0532-247618.
E-mail address: cpa@unife.it (A. Caputo).

0264-410X/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2003.12.025

or mucosal administration [18], a common problem with this type of delivery systems is the instability or the degradation of the entrapped antigen. This may occur either during the encapsulation process, such as the exposure to organic solvents, high shear and freeze-drying, and/or in the body when the antigen is exposed to the low pH microenvironment caused by the degradation of the polymer [1,19,20].

To overcome these problems and to develop new and improved protein vaccine delivery systems, characterized by an increased shelf-life and low costs, novel biocompatible polymeric microspheres capable of reversibly adsorbing native proteins at their surface, were synthesized by dispersion polymerization [21]. These microspheres have a core-shell structure constituted by a soft outer shell, made of long soluble arms able to fix the protein, anchored to an inner hard insoluble core. In particular, two classes of negatively charged microspheres, either made of poly(styrene) (PS) or poly(methyl methacrylate), and in which the steric stabilizers are hemisuccinated poly(vinyl alcohol) or Eudragit L100/55, respectively, were prepared. Since recent studies have indicated that the HIV-1 Tat protein represents a promising candidate of a prophylactic and/or therapeutic vaccine against AIDS, and since Tat contains a positively charged domain, rich in arginine and lysine [22–26], we investigated whether these novel anionic microspheres were capable of reversibly adsorbing a biologically active HIV-1 Tat protein, preserving its native conformation, biological activity, and its shelf-life, and efficiently delivering it intracellularly. In addition, in view of their potential development as new delivery systems for vaccine application, their safety was studied both in vitro and in vivo. The results indicate that the novel anionic microspheres preserve Tat conformation and activity, and efficiently deliver the protein into the cells, in the absence of in vitro or in vivo toxicity. Therefore, they are suitable storage and delivery systems for vaccine applications, particularly when the native protein conformation is required.

2. Materials and methods

2.1. Microspheres

Benzoyl peroxide (BPO), poly(vinyl alcohol) (molar mass 49.000), styrene, succinic anhydride, methyl methacrylate were purchased from Sigma–Aldrich (St. Louise, MI). Poly(methacrylic acid, ethyl acrylate) 1:1 statistical copolymer powder (trade name Eudragit L100/55; average molar mass of 250.000 g/mol) was supplied by Röhm GmbH (Darmstadt, Germany). Samples A4 and A7 were prepared by dispersion polymerization of styrene (monomer) in the presence of hemisuccinated poly(vinyl alcohol) as the steric stabilizer, as described previously [21]. Samples 1D, 1E, H1D were obtained by dispersion polymerization of methyl methacrylate (monomer) in the presence of Eudragit L100/55 as the steric stabilizer. H1D fluorescent microspheres were produced similarly by dispersion polymerization in the presence of a newly prepared fluorescein-based allylic monomer of methyl methacrylate. Briefly, the preparation of the microsphere A7 was as follows: 1.86 g of hemisuccinated poly(vinyl alcohol), 15.5 ml of styrene, 1.95 g of BPO were dissolved in 162 ml of ethanol/2-methoxyethanol 1/1 under a nitrogen atmosphere. The solution was heated at 70 °C for 48 h under mechanic stirring (60 rpm). The reaction mixture was then cooled and, after three cycles of centrifugation and redispersion with the organic solvent and two cycles with HPLC grade water, the resulting particles were lyophilized. A 76% yield was obtained. Similarly, A4 microspheres were prepared starting from 1.34 g of hemisuccinated poly(vinyl alcohol) dissolved in 162 ml of ethanol/2-methoxyethanol 9/1 (yield = 82%). As concerned the Eudragit stabilized poly(methyl methacrylate) microspheres, as a typical example, the preparation of sample 1D was as follows: 14.73 g of Eudragit were dissolved under a nitrogen atmosphere for 30 min in methanol heated at 60 °C. α,α'-azoisobutyronitrile (0.37 g) was dissolved in 18.4 g of methyl methacrylate monomer and added to the solution. The reaction was left to proceed for 24 h under constant stirring. The reaction mixture was then cooled and, after three cycles of centrifugation and redispersion with methanol followed by two cycles with deionized water, the resulting particles were lyophilized. A 70% yield was obtained. In a fashion similar to the synthesis of A4 and A7, the variation in size and surface charge density of 1D, 1E, H1D and fluorescent-H1D was obtained using different amounts of steric stabilizer and solvent composition during the synthesis. Synthesis details will be described elsewhere [Sparnacci et al., personal communication]. Microspheres can be stored lyophilized at room temperature or resuspended (2 mg/ml) in degassed sterile phosphate buffered saline (PBS) at 4 °C.

2.2. Particle size and morphology analysis

Particle size was measured by a Jeol JEM-100CX scanning electron microscope (SEM) (Akishima, Japan) at an accelerating voltage ranging between 20 and 30 kV. The samples were sputter-coated with gold. The amount of steric stabilizer covalently linked to the microsphere surface was determined by acid–base titration [Sparnacci et al., personal communication].

2.3. Adsorption of Tat to the microspheres

The biologically active Tat protein of HIV-1 (HTLVIII-BH10) was produced in *Escherichia coli*, purified and tested for activity as previously described [27–29]. To prevent oxidation that occurs easily because Tat contains seven cysteines, the Tat protein was stored lyophilized at −80°, and resuspended in degassed sterile PBS (2 mg/ml) immediately before use, as described [27–29]. In addition, since Tat is photo- and thermo-sensitive, the handling of Tat was always performed in the dark and on ice. Experiments were also performed with Tat oxidized by exposure to light and air for 16 h. By this procedure, Tat loses its biological activity due to conformational changes, including multimerization and aggregation of the protein with loss of the monomeric active form [27–29]. Endotoxin concentration of different lots of Tat was always below the detection limit (<0.05 EU/μg), as tested by the *Limulus* Amoebocyte Lysate analysis. The appropriate volumes of Tat and microspheres were mixed in 200 μl of PBS and incubated in the dark and on ice for 60 min. In some experiments, samples were exposed to air and light at room temperature for 16 h. In both cases, after incubation samples were spun at 13,000 rpm for 10 min. The pellets (Tat–microspheres complexes) were resuspended in the appropriate volume of degassed sterile PBS and used immediately.

2.4. Flow cytometry

Microspheres (50 μg) were incubated with increasing amount of the Tat protein (0.1, 0,5, 1, 2, 5 and 10 μg) in a final volume of 50 μl for 60 min at room temperature under mild agitation. Microspheres alone or microsphere–Tat complexes were spun at 13,000 rpm for 15 min, washed twice and resuspended in 50 μl of PBS. Five μl of microspheres–Tat complexes or microspheres alone were then incubated for 30 min at 4 °C with a FITC-labeled anti-Tat rabbit polyclonal antibody [Magnani, et al., unpublished results] and analyzed by flow cytometry (FacScan Becton-Dickinson Mountain View, CA).

2.5. Cell cultures

Monolayer cultures of human HL3T1 cells, containing an integrated copy of plasmid HIV-1-LTR-CAT, where expression of the chloramphenicol acetyl transferase (CAT) reporter gene is driven by the HIV-1 LTR promoter, were obtained through the NIH AIDS research and reference reagents program (Bethesda, MD) and grown in DMEM (Gibco, Grand Island, NY) containing 10% FBS (Gibco).

2.6. Isolation of murine and human primary cells

Six-week-old Swiss female mice (Nossan, Italy) were injected intraperitoneally (i.p.) with 1.0 ml of 10% thioglycolate (Sigma). At 4 days, mice were sacrificed, and peritoneal exudates cells highly enriched for macrophages were harvested by i.p. lavage with 10 ml of ice-cold Hank's balanced salt solution supplemented with 10 U/ml of heparin. Cells (4×10^6 cells) were washed twice, resuspended in DMEM supplemented with 10% heat-inactivated FBS, 1% antibiotics, 2 mM glutamine, seeded onto 35 mm Petri dishes, and incubated for 12 h in a humidified 5% CO_2 atmosphere at 37 °C to allow macrophage adherence. No adherent cells were gently removed with warmed DMEM medium. Monolayers were 95% pure macrophages as determined by immunostaining and surface marker analysis using a rat monoclonal antibody to mouse F4/80 (Caltag Lab., Burlingame, CA).

Murine splenocytes were purified from spleens of 10-week-old BALB/c female mice using Ficoll gradients, as described [30], and grown in RPMI 1640 supplemented with 10% FBS. Human monocytes and monocyte-derived dendritic cells were purified from a buffy coat, characterized and cultured as described [31].

2.7. Analysis of cytotoxicity in vitro

HL3T1 cells (1×10^4/100 μl) were seeded in 96-well plates and cultured at 37 °C for 24 h. One-hundred microliter of medium containing the microspheres alone (10, 30, 50, 100, 300, 500 and 1000 μg/ml) or bound to Tat (1 μg/ml) (sextupled wells) were then added to the cells. Untreated cells and cells incubated with Tat alone were the controls. Cells were incubated for 96 h at 37°, and cell proliferation was measured using the colorimetric cell proliferation kit I (MTT-based) provided by Roche (Roche, Milan, Italy) [32].

2.8. Cellular uptake of microspheres

HL3T1 cells (1×10^5) were seeded in 24-well plates containing a 12 mm glass coverslip, and incubated with fluorescent-H1D microspheres. After incubation, cells were washed, fixed with 4% cold paraformaldehyde and observed at a confocal laser scanning microscope LSM410 (Zeiss, Oberkochen, Germany). Image acquisition, recording and filtering were carried out using a Indy 4400 graphic workstation (Silicon Graphics, Mountain View, CA) as previously described [33].

Human monocytes and monocyte-derived dendritic cells (1×10^5), and murine splenocytes (4×10^6) were incubated in 24-well plates with fluorescent-H1D microspheres for 24 h. After incubation, cells were washed and layered onto glass slides previously coated with poly-L-lysine (Sigma) according to manufacturer's instructions. Cells were fixed with 4% cold paraformaldehyde, stained with 4′-6′-diamidino-2-phenylindole (DAPI; Sigma) and observed with a confocal microscope, as described above, and at a fluorescent microscope Axiophot 100 (Zeiss). The green fluorescence (microspheres) was observed with a 450–490λ, flow through 510λ and long pass 520λ filter; the blue fluorescence (DAPI) was observed with a band pass 365λ, flow through 395λ and long pass 397λ filter. For the same microscopic field, green, blue and phase contrast images were taken with a Cool-Snapp CCD camera (RS-Photometrics, Fairfax, VA). The three images were then overlapped using the Adobe Photoshop 5.5 program.

Murine macrophages (3×10^6) were incubated in the presence of microspheres, at a ratio of 4 microspheres per macrophage, for 1, 2 and 4 h. Cells were extensively washed to remove non-phagocytosed microspheres, fixed with 2% paraformaldehyde and 2.5% glutaraldehyde for 30 min at

4 °C, and stained with toluidine blue. Cells were observed at a phase contrast microscope (100×) to count the number of macrophages with phagocytosed microspheres. The percentage of cellular uptake was calculated as follows: [mean number of cells with phagocytosed microparticles/mean total number of cells] × 100, counted in three microscopic fields.

2.9. Immunofluorescence

HL3T1 cells (1×10^5) were seeded in 24-well plates containing a 12 mm glass coverslip, and incubated with fluorescent-H1D microspheres–Tat protein complexes. The dose of 30 μg/ml of miscrospheres associated with 5 μg/ml of Tat was used. Controls were represented by cells incubated with the Tat (5 μg/ml) protein alone or untreated cells. After incubation, cells were washed, fixed with 4% cold paraformaldehyde and analyzed by immunofluorescence with an anti-Tat monoclonal antibody (4B4C4) and a goat Cy3-conjugated anti-mouse IgG secondary serum, as previously described [34]. Cells were colored with DAPI and observed at a fluorescence microscope. The red fluorescence (Tat) was observed with a band pass 546λ, flow through 580λ and long pass 590λ filter; the green (microspheres) and blue fluorescence (DAPI) were observed as described above. For the same microscopic field, green, red, blue and phase contrast images were taken and overlapped as described above.

2.10. Gel electrophoresis

Microspheres (50 μg) were incubated with increasing amounts of the Tat protein (1, 2, 5, 10 μg) in a final volume of 50 μl for 60 min at room temperature under mild agitation. Microsphere–Tat complexes were spun at 13,000 rpm for 10 min, washed three times with PBS, and resuspended in 25 μl of 0.5 M Tris/HCl, pH 6.8, containing SDS 2%, MSH 4% (v/v) and bromophenol-blu (sample buffer). Samples were boiled for 10 min and spun at 13,000 rpm for 10 min. Supernatants (recovered Tat) were run onto 14% SDS-PAGE and stained with Coomassie blue [35]. Free Tat protein (1, 2, 5 and 10 μg) was resuspended in 25 μl of sample buffer and run in each gel as the standard control. Gels were analysed with a GelDoc Quantity One system (BioRad, München, Germany), and the amount of Tat recovered after boiling was determined by linear regression analysis on the Tat standard curve included in each gel. The microsphere loading ability (w/w) is determined as follows: [Tat (recovered) (μg)/microspheres used to form the complexes (50 μg)] × 100.

2.11. Evaluation of the Tat protein activity

Evaluation of Tat protein activity was performed using HL3T1 cells. These cells contain an integrated copy of the bacterial CAT reporter gene whose expression is driven by the HIV-1 LTR-promoter. In these cells expression of CAT occurs only in the presence of bioactive Tat protein and it correlates with the amount of Tat. For this purpose, HL3T1 cells (5×10^5) were seeded in 60 mm Petri dishes, and 24 h later cells were replaced with 1 ml of fresh medium and incubated with Tat alone (0.1, 0.25, 0.5, 1 μg/ml) or Tat adsorbed onto the microspheres (30 μg/ml) in the absence or presence of 100 μM chloroquine (Sigma). In some experiments, before the addition to the cells, Tat alone or Tat–microsphere complexes were exposed to air and light at room temperature for 16 h. CAT activity was measured 48 h later in cell extracts after normalization to total protein content, as described previously [34,35]. The percentage of CAT activity was calculated by the formula [cpm of the acetylated ^{14}C-chloramphenicol/total cpm of acetylated and unacetylated ^{14}C-chloramphenicol] × 100.

2.12. Safety studies

Animal use was according to national and institutional guidelines. BALB/c mice (7–8-week-old female) (Nossan, Milan, Italy) were injected with 30 μg of microspheres bound to the Tat protein (0.5 or 2 μg). Control mice were injected with the Tat protein alone, Tat protein in Freund's adjuvant (CFA for the first inoculation, IFA for subsequent inoculations), or PBS. Samples (100 μl) were given by intramuscular (i.m.) injections in the quadriceps muscles of the posterior legs. Mice were injected twice (protocol 1), at weeks 0 and 2, and in each group half number of mice were sacrificed 2 weeks after the first injection, and the remaining animals 2 weeks after the second injection. Alternatively, mice were injected three times (protocol 2), at weeks 0, 4 and 8, and in each group half number of mice were sacrificed 2 weeks after the second injection, and the remaining animals 2 weeks after the third injection. During the course of the experiments, animals were controlled twice a week at the site of injection, for the presence of edema, induration, redness, and for their general conditions, such as liveliness, vitality, weight, motility, sheen of hair. At sacrifice mice were anesthetized i.p. with 100 μl of isotonic solution containing 1 mg of Inoketan (Virbac, Milan, Italy), and 200 μg Rompun (Bayer, Milan, Italy).

2.13. Histological, histochemical and immunohistochemical procedures

At sacrifice animals were subjected to autopsy. Samples of cutis, subcutis and skeletal muscles at the sites of injection and other organs (lungs, heart, intestine, kidneys, spleen and liver) were fixed in 10% formalin for 12–24 h, embedded in paraffin, and routinely processed for histological examination. Paraffin-embedded sections (3–5 μm) were stained with hematoxylin and eosin, subjected to periodic acid-Shiff (PAS) reaction with and without diastase treatment (Sigma). Serial tissue sections were immune-stained using the avidin–biotin–peroxidase complex technique

(Vectastain ABC Kit PK-4002, Vector Labs, Burlingame, CA) according to Hsu et al. [36]. The panel of antibodies included S-100 (Dako, Denmark), HH-F 35 (Dako) for detection of α-actin, CD68 and Mac387 (Dako) for detection of macrophages. Briefly, after deparaffinization and rehydration, endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol; samples were then incubated with primary antibodies for 10-12 h at 4 °C. Biotinilated-anti-mouse and anti-rabbit immunoglobulins (Sigma) were utilized as secondary antibodies. Specific reactions were detected following incubation with avidin–biotin–peroxidase conjugated and treatment with diaminobenzidine (Sigma) and hydrogen peroxide.

2.14. Statistical analysis

Student's *t*-test was performed as described [37].

3. Results

3.1. Polymeric microspheres adsorb the HIV-1 Tat protein at their surface

Two novel classes of biocompatible polymeric anionic microspheres, made of poly(styrene) or poly(methyl methacrylate) (PMMA) and in which the steric stabilizers are hemisuccinated poly(vinyl alcohol) and Eudragit L100/55, respectively, were synthesized by dispersion polymerization. The particles appeared spherical, smooth and homogeneous in size (Fig. 1). In both classes of microspheres the long soluble arms of the outer shell are covalently bound to the surface of the particles. This feature distinguishes these novel particles from other colloidal systems whose surface is simply coated by hydrophilic polymers [38,39]. In this study, five types of microparticles, two made of PS (A4 and A7) and three made of PMMA (1D, 1E, H1D) were chosen for characterization as protein delivery systems for vaccine applications.

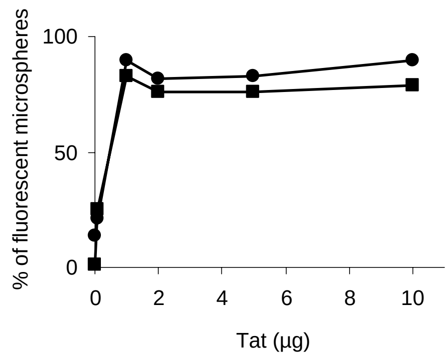

Fig. 2. Flow cytometry analysis of Tat adsorbed to the surface of polymeric microsphere. One representative type of microsphere for each class is shown: A7 (●) made of poly(styrene) and hemisuccinated poly(vinyl alcohol) and 1E (■) made of poly(methyl methacrylate) and Eudragit L100/55.

Microspheres A4 (0.99 ± 0.03 μm) and A7 (3.46 ± 0.10 μm) are made of an inner hard core of poly(styrene) and an outer shell of negatively charged groups derived from hemisuccinated poly(vinyl alcohol) stabilizer. Microspheres 1D (4.35 ± 1.02 μm), 1E (2.60 ± 0.45 μm), H1D (1.69 ± 0.16 μm) and fluorescent-H1D (2.13 ± 0.09 μm) are composed of an inner hard core of poly(methyl methacrylate) and an outer shell of negatively charged groups derived from Eudragit L100/55 stabilizer. In addition, all microspheres differ for their surface negative charge density (Table 1).

To determine whether the HIV-1 Tat protein could bind to the surface of these microparticles, Tat was incubated with A4, A7, 1D, 1E and H1D samples to allow adsorption, and the Tat–microsphere complexes were then analyzed by flow cytometry. The results indicated that Tat adsorbs at the surface of both type of anionic microspheres (Fig. 2). Although

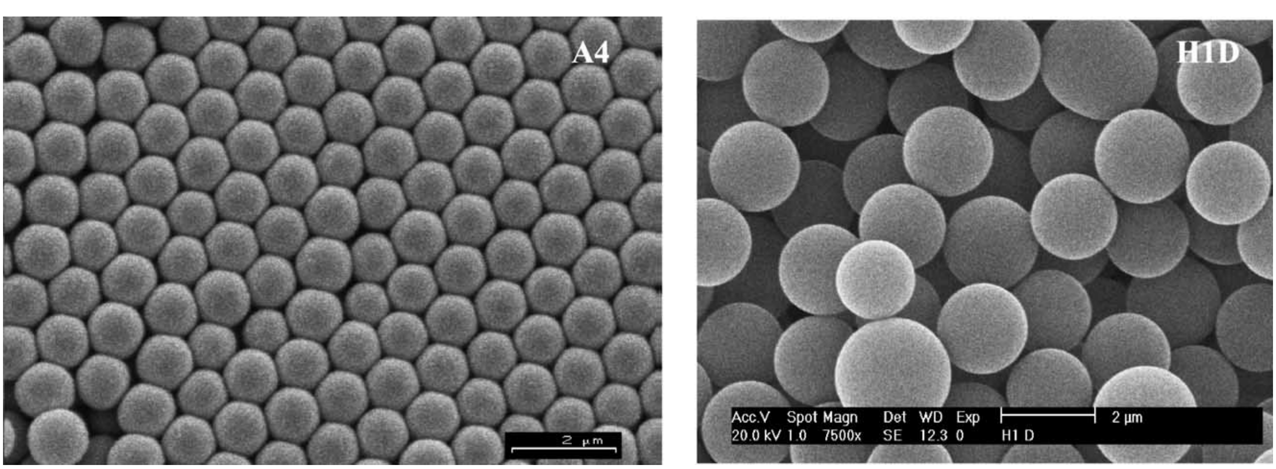

Fig. 1. SEM image of polymeric anionic microparticles produced by dispersion polymerization. One representative type of microparticle for each class is shown: A4 made of poly(styrene) and poly(vinyl alcohol) stabilizer, and H1D made of poly(methyl methacrylate) and Eudragit L100/55 stabilizer.

Table 1
Physical properties of polymeric microspheres[a]

Microsphere	Polymer (stabilizer)	Diameter (μm)	COOH/microsphere (μmol COOH/g)
A4	PS (EPVA)	0.99 ± 0.03	37.4
A7	PS (EPVA)	3.46 ± 0.10	20.1
1D	PMMA (Eudragit L100/55)	4.35 ± 1.02	48.1
1E	PMMA (Eudragit L100/55)	2.60 ± 0.45	59.2
H1D	PMMA (Eudragit L100/55)	1.69 ± 0.16	62.1
H1D fluorescent	PMMA (Eudragit L100/55)	2.13 ± 0.09	59.2

[a] Physical properties of polymeric microspheres composed of an inner hard core made of poly(styrene) (PS) or poly(methyl methacrylate) (PMMA), and of carboxyl (COOH) functional surface groups derived, respectively, from hemisuccinated poly(vinyl alcohol) (EPVA) or Eudragit L100/55 stabilizers. Microspheres were synthesized as described in Section 2.

the maximum fluorescence, which represents the percentage of microspheres that bind Tat, was already detected with 1 μg of Tat, this result is not quantitative, likely because of antibody steric hindrance. A more quantitative analysis of the Tat protein adsorption onto the particle surface was performed by SDS-PAGE. In these experiments, the same dose of microspheres (50 μg) was mixed with increasing amounts of Tat (1, 2, 5 and 10 μg). Then, Tat bound to the microspheres was dissociated from each complex and analysed by gel electrophoresis. As shown in Fig. 3, Tat binds on the microparticle surface in a dose-dependent fashion. The microsphere loading ability (w/w) ranges between 0.2 and 9.3% according to the amount of added Tat.

Finally, both classes of microparticles were stable and could be stored lyophilized or as suspension, as described in the methodology section, for several months. Changes in terms of their capacity to adsorb (and to release) Tat have

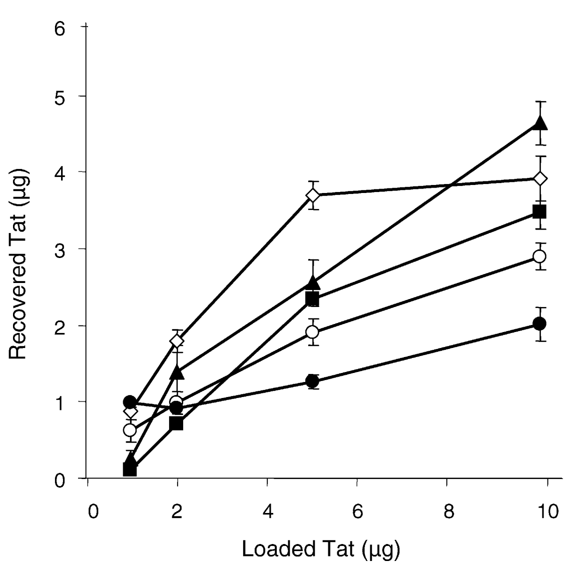

Fig. 3. Loading of Tat to the microsphere surface. Microspheres (50 μg) were mixed with increasing amounts of Tat (1, 2, 5, 10 μg), and extensively washed. Tat was recovered from the complexes, analysed by SDS-polyacrylamide gel electrophoresis and quantified as described in the methodology section. Data are the mean of three different experiments (±S.D.). A4 (◇) and A7 (○) made of poly(styrene) and hemisuccinated poly(vinyl alcohol); H1D (▲), 1E (■) and 1D (●) made of poly(methyl methacrylate) and Eudragit L100/55.

been found in microspheres suspensions stored at + 4 °C after more than 8 months.

3.2. Measurement of in vitro cytotoxicity

An important requirement of synthetic delivery systems is the lack of cytotoxicity. Possible cytotoxic effects may depend on the chemical composition, charges, size dispersion, and dose of the microspheres. To test whether the novel polymeric microspheres composed of poly(styrene) (A4, A7) and of poly(methyl methacrylate) (1D, 1E and H1D) are cytotoxic, HL3T1 cells were incubated with increasing concentrations (10–1000 μg/ml) of each microsphere, alone or bound to Tat (1 μg/ml). After 96 h incubation, cell proliferation was measured by the MTT assay. As shown in Fig. 4, both classes of microspheres and microsphere–Tat complexes were not toxic to the cells up to 50 μg/ml as compared to untreated or Tat-treated cells ($P < 0.01$); a 50% reduction of cell viability was observed only at higher doses (300–1000 μg/ml) (data not shown). Based on these results we have chosen the dose of 30 μg for all subsequent in vitro and in vivo studies.

3.3. Phagocytosis of microspheres

Phagocytosis of microspheres by antigen-presenting cells is controlled by several factors including size, surface chemistry and morphology of the particles [40–42]. Thus, we analyzed whether the polymeric microspheres were phagocyted by different types of cells, such as professional phagocytes and epithelial cells. Experiments in murine macrophages, cultured in the presence of both types of microspheres and analyzed at a phase contrast microscope, indicated that all particles were taken up with similar kinetics and percentage of phagocytosis (Fig. 5). Similar results were obtained when fluorescent-H1D were added to human monocytes, monocyte-derived dendritic cells, murine splenocytes and HL3T1 cells, and observed with confocal and fluorescent microscopy. The results shown in Fig. 6, indicate internalization efficiency ranging from 12% for murine splenocytes, 18-20% for human monocyte-derived dendritic cells and 25–30% for human monocytes and HeLa cells. These data

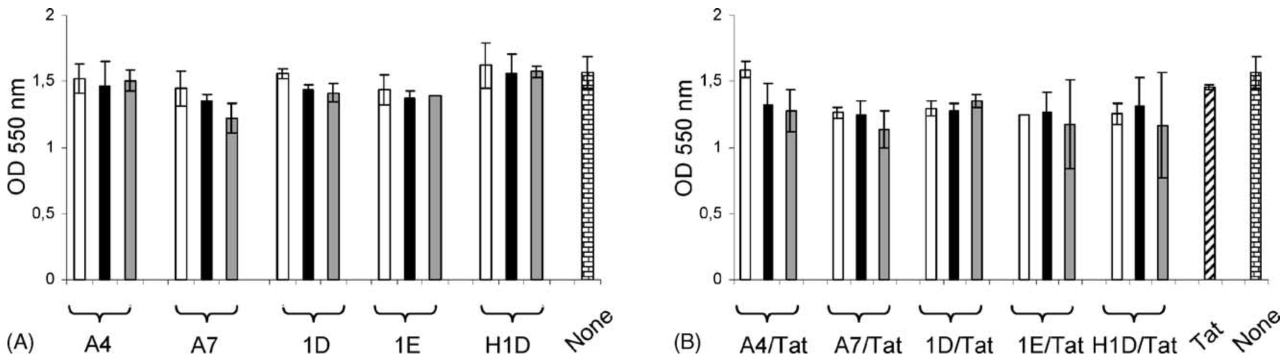

Fig. 4. Evaluation of cell proliferation in the presence of the microspheres alone or the Tat–microsphere complexes. HL3T1 cells were cultured for 96 h with 10 μg/ml (empty bars), 30 μg/ml (black bars), and 50 μg/ml (gray bars) of microspheres alone (A), or with the same doses of microspheres bound to Tat (1 μg/ml) (B). Controls were represented by untreated cells (None) or cells cultured with 1 μg/ml of Tat (Tat).

indicated that the microparticles are taken up by different cell types and that chemical composition and size do not affect their phagocytosis.

3.4. Tat–microsphere complexes enter the cells and release Tat in a controlled fashion

Extracellular Tat protein can be taken up by cells after interaction with heparan–sulfate proteoglycans and integrin receptors at the cell surface [28,29,43]. To determine whether intact Tat–microsphere complexes enter the cells and release Tat intracellularly, or whether Tat is delivered at the cell surface and then released from the Tat–microsphere complexes and taken up by the cells, HL3T1 cells were incubated with Tat bound to fluorescent-H1D microspheres and analyzed by immunofluorescence with an anti-Tat mAb. The results indicated that the Tat–microsphere complexes are readily taken up by the cells and release Tat intracellularly in the proximity of the nucleus (Fig. 7). Of note, Tat is released in a controlled fashion, as suggested by the observation that after 48 h Tat-loaded particles are still detectable in the cells (Fig. 7).

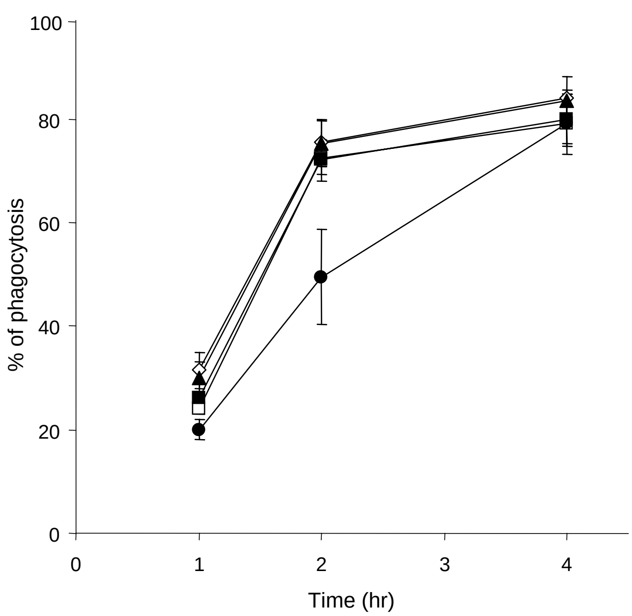

Fig. 5. Murine macrophages phagocyte polymeric microparticles. Murine macrophages were cultured with microspheres, fixed, colored with toluidine blue and observed at a phase contrast microscope. Results are expressed as the percentage of cells that phagocyted the microspheres, and they are the mean of three different experiments (±S.D.). A4 (◇) and A7 (○) made of poly(styrene) and hemisuccinated poly(vinyl alcohol); H1D (▲), 1E (■) and 1D (●) made of poly(methyl methacrylate) and Eudragit L100/55.

3.5. Polymeric microspheres protect HIV-1 Tat from oxidation

Tat protein oxidizes very easily with air and light and it is labile at room temperature due to the presence of seven cysteines in its sequence [26]. Oxidation leads to protein multimerization, aggregation and loss of the biological activity, which requires a native protein conformation. Therefore, special procedures must be followed for purification, handling and storage of Tat in order to preserve its native conformation [27–29]. Of note, both the immunomodulatory effects of Tat on macrophage-derived dendritic cells, and protection of monkeys vaccinated with Tat from a pathogenic challenge were observed utilizing a Tat protein in the native conformation and fully biologically active [22–24,29]. Therefore, the best evidence for Tat protein integrity is to assess its biological activity. To determine whether Tat bound to the microspheres was protected from oxidation, Tat–microsphere complexes or Tat alone were insufflated with air and exposed to light for 16 h at room temperature before the addition to the HL3T1 cells. CAT activity was then compared to that induced by untreated native Tat. The results, shown in Fig. 8, indicate that the exposure to air and light did not inactivate Tat trans-activating function when Tat was previously adsorbed onto the microspheres, whereas it caused the loss of Tat biological activity when Tat was free. Thus, Tat bound to the microspheres was protected from oxidation. This result was confirmed in a different set of experiments in which Tat, free or bound

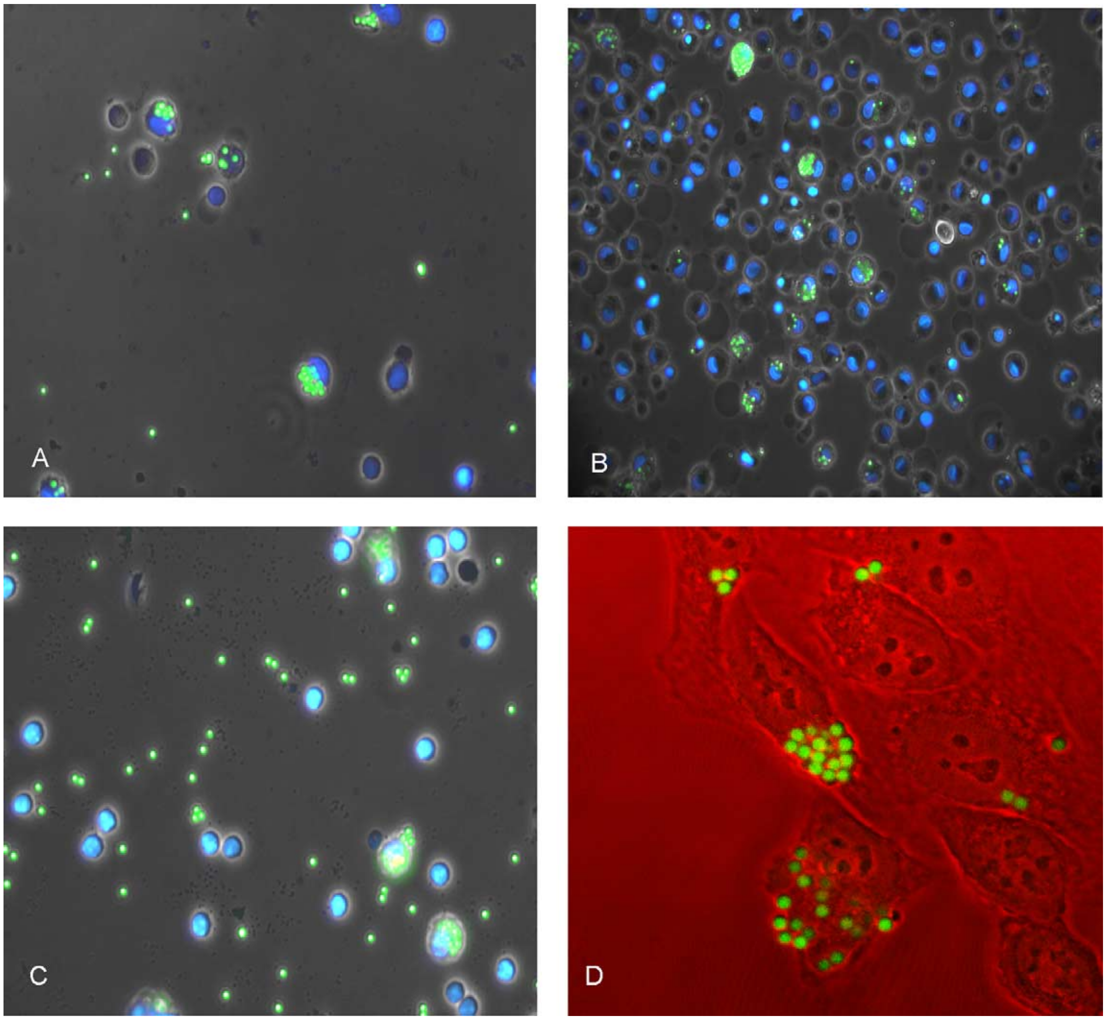

Fig. 6. Analysis of microspheres uptake. Human monocytes (A), monocyte-derived dendritic cells (B), murine splenocytes (C) and HL3T1 cells (D) were cultured in the presence of fluorescent H1D microparticles for 24 h, fixed with paraformaldheyde and observed at fluorescent and confocal microscopes. Representative images of fluorescent microscopy are shown in panels A–C, and of confocal microscopy in panel D.

to the microspheres, was analyzed by SDS-PAGE gel electrophoresis either before and after exposure to light and air at room temperature. Exposure of free Tat to oxidizing conditions caused the decrease of the monomeric bioactive form of Tat and, concomitantly, the increase of oxidized Tat multimers, as compared to free Tat not exposed to air and light (data not shown). In contrast, when Tat was bound to the microspheres, the monomeric conformation of Tat was the most abundant form, either before or after exposure to air and light (data not shown).

3.6. Polymeric microspheres bind and release biologically active Tat protein

For their application as delivery systems in vaccine development, polymeric microspheres should bind and release a protein in its biologically active conformation. This is particularly important for Tat since a native protein is required for vaccine efficacy [22–24]. Therefore, the capability of the microspheres to bind and release the HIV-1 Tat protein in its biologically active conformation was determined in HL3T1 cells, containing an integrated copy of the reporter plasmid HIV-1 LTR-CAT. Cells were incubated with increasing amounts of Tat alone or Tat adsorbed onto A4, A7, 1D, 1E and H1D microspheres. Expression of CAT was maximal and similar among all Tat–microsphere complexes (Fig. 9). In addition, at the doses of 100, 250 and 500 ng/ml of Tat bound to the microspheres, CAT expression was significantly higher than that elicited by the same doses of Tat alone (Fig. 9), suggesting that Tat bound at the surface of the microspheres is protected from proteolytic degradation and/or released in a controlled fashion from the complexes, in agreement with the previous results shown earlier (Fig. 7). These results demonstrate that all the microspheres tested adsorb and release biologically active Tat protein in a dose-dependent fashion, and that Tat bound to the microspheres maintains its native conformation and biological activity.

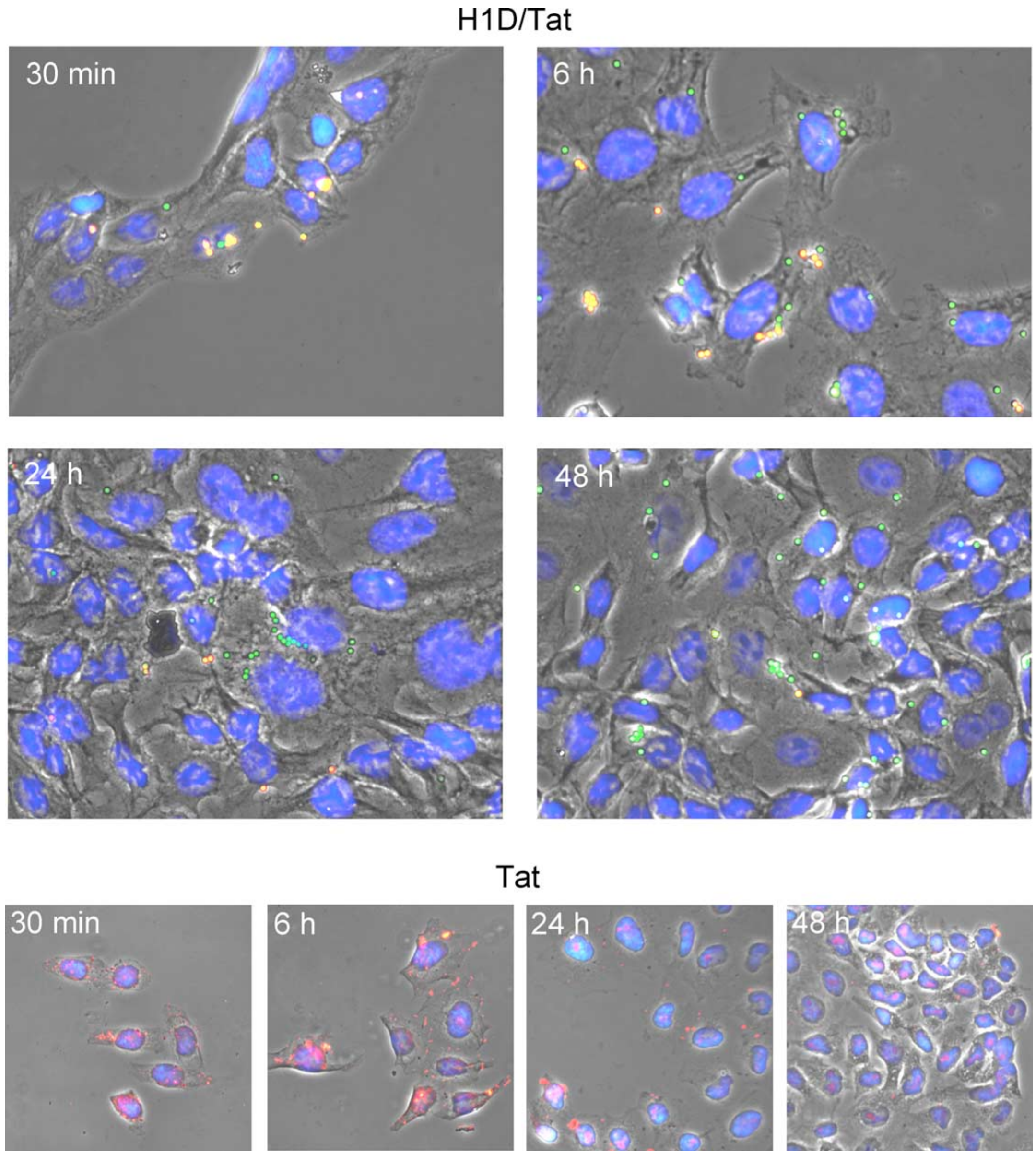

Fig. 7. Polymeric microspheres deliver and release HIV-1 Tat intracellularly. HL3T1 cells were cultured in the presence of fluorescent-H1D (30 μg/ml) bound to Tat (5 μg/ml), or with Tat alone (5 μg/ml), fixed and analyzed by immunofluorescence using an anti-Tat monoclonal antibody. For the same microscopic field, green (H1D), red (Tat), blue (DAPI) and phase contrast (cells) images were taken with a CCD camera and overlapped with a Adobe Photoshop program.

3.7. Evaluation of the safety of Tat–microsphere complexes in vivo

To study the safety of these novel microparticles in vivo, mice ($n = 176$) were injected with the Tat–microsphere complexes. Control mice ($n = 120$) were injected with Tat alone, Tat and Freund's adjuvant, or PBS. Animals were injected at weeks 0 and 2 (protocol 1), or at weeks 0, 4 and 8 (protocol 2) (Table 2). During the course of the experiment, each animal was controlled twice a week at the site of injection and for its general health conditions. No signs of local nor systemic adverse reactions were ever observed in mice receiving the Tat–microsphere complexes, as compared to control mice injected with Tat or with PBS. Mice were sacrificed after the first and second injection in protocol 1, and after the second and third inoculation in protocol 2, and tissues and organs were collected for histological and immunohistochemical examination.

Two types of histological pictures were observed at the site of injection. The first consisted of small foci, involving one or two muscle fibers, showing increased number of nuclei, and scarce macrophage infiltrate in the interstitial space (Fig. 10A and C). These features were prevalently detected in mice injected with the Tat–microsphere complexes or Tat

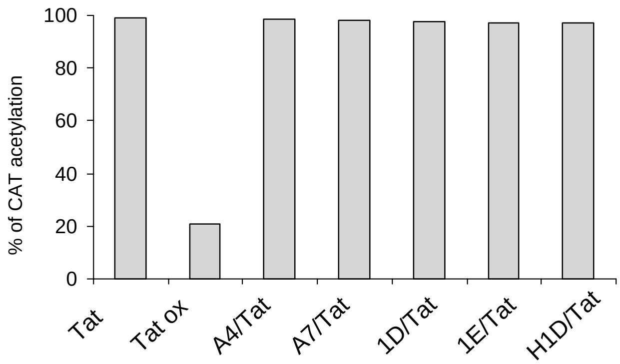

Fig. 8. Polymeric microspheres protect HIV-1 Tat from oxidation. HL3T1 cells, containing an integrated copy of the reporter vector HIV-1 LTR-CAT, were incubated with Tat (1 μg/ml) adsorbed to the microspheres (30 μg/ml) and exposed to air and light for 16 h at room temperature. Control cells were incubated with the same dose of the protein, which was untreated (Tat) or oxidized by exposure to air and light (Tat ox). The percentage of CAT activity was calculated as described [34]. Results are the mean of two independent experiments.

alone. The second type of picture was found in the muscular fascia and in the surrounding adipose tissue, and it was characterized by a central area of necrosis surrounded by neutrophil granulocytes and macrophages (Fig. 10B and D). The macrophages always showed good reactivity to CD68 and Mac387 monoclonal antibodies; T and B lymphocytes were not detected in the inflammatory reactions. This type of lesion, as well as the higher number of inflammatory cells, was detected in the majority of mice receiving Tat and Freund's adjuvant. In the other animals and in control mice inoculated with PBS, the inflammatory reaction was inconspicuous, related to the traumatic stimulus or absent (Table 3). Laden macrophages reaction or other type of inflammatory reactions were not observed in the other organs.

Table 2
Scheme of inoculation[a]

Sample	Dose (Tat/microsphere)	Protocol 1 mice injected	Protocol 2 mice injected
Tat/A4	0.5 μg/30 μg	16	10
	2 μg/30 μg	6	50
Tat/A7	0.5 μg/30 μg	16	10
	2 μg/30 μg	6	n.d.[b]
Tat/1D	0.5 μg/30 μg	10	10
Tat/1E	0.5 μg/30 μg	10	10
Tat/H1D	0.5 μg/30 μg	10	10
Tat/Freund's	0.5 μg	16	10
	2 μg	6	n.d.[b]
Tat	0.5 μg	6	n.d.[b]
	2 μg	6	n.d.[b]
PBS	None	16	60

[a] BALB/c female mice were injected i.m. with 0.5 or 2 μg of Tat adsorbed to 30 μg of microspheres, Tat and Freund's adjuvant, Tat alone or PBS, at weeks 0 and 2 (protocol 1) or at weeks 0, 4 and 8 (protocol 2). The number of mice injected with each sample is reported.

[b] n.d.: not done.

Table 3
Tat–microsphere complexes induce a poor inflammatory reaction at the site of injection[a]

Group	Mice with inflammatory reaction at the site of injection/number of mice examined		
	I	II	III
Tat/A4	1/8 (12)	7/17 (41)	10/16 (62)
Tat/A7	0/8 (0)	2/12 (17)	3/6 (50)
Tat/1D	1/2 (50)	2/6 (33)	4/6 (66)
Tat1E	0/2 (0)	2/6 (33)	3/6 (50)
Tat/H1D	0/2 (0)	1/6 (17)	3/4 (75)
Tat/Freund's[b]	6/8 (75)	11/12 (91)	6/6 (100)
Tat	0/6 (0)	3/6 (50)	n.d.[c]
PBS	0/6 (0)	0/6 (0)	0/10 (0)

[a] BALB/c mice were injected at weeks 0 and 2 or 0, 4 and 8, and sacrificed 2 weeks after the first (I), second (II) or third (III) immunization, respectively, for histological and immunohistochemical examination of the muscle at the site of injection and of other organs. Percentage of mice developing an inflammatory reaction at the site of injection are reported in parenthesis.

[b] All mice injected with Tat and Freund's adjuvant developed a granuloma at the site of injection that was visible a few days after the first inoculation.

[c] n.d.: not done.

No differences in the inflammatory reactions, related to the chemical composition and size of microspheres or the dose of Tat, were detected after one immunization. Indeed, only 2/22 (9%) mice, inoculated with A4-Tat 0.5 μg or 1D-Tat 0.5 μg, showed an inflammatory reaction. After two immunizations, 14/47 (30%) mice treated with the microsphere–Tat complexes developed a local inflammatory reaction. After three immunizations, 23/38 (60%) of mice treated with the Tat–microsphere complexes showed variable inflammatory reactions at the site of inoculation. In conclusion, the frequency of the inflammatory reactions correlated with the number of immunizations.

Tat-treated mice presented local inflammation (type one picture) only after the second inoculation in about 50% of the mice; macrophages infiltration was more frequently observed, but it was not related to the dose of Tat.

All mice treated with Tat and Freund's adjuvant showed intense inflammatory reactions independently from the number of immunizations; the incidence was more than 70% after the first injection and raised up to 90–100% after the second and the third treatment. This is likely due to the type of adjuvant used.

4. Discussion

Several microspheres with different polymer composition, poly(methyl methacrylate) or poly(styrene), different surface functionalization and size were prepared by dispersion polymerization and characterized. In view of their possible

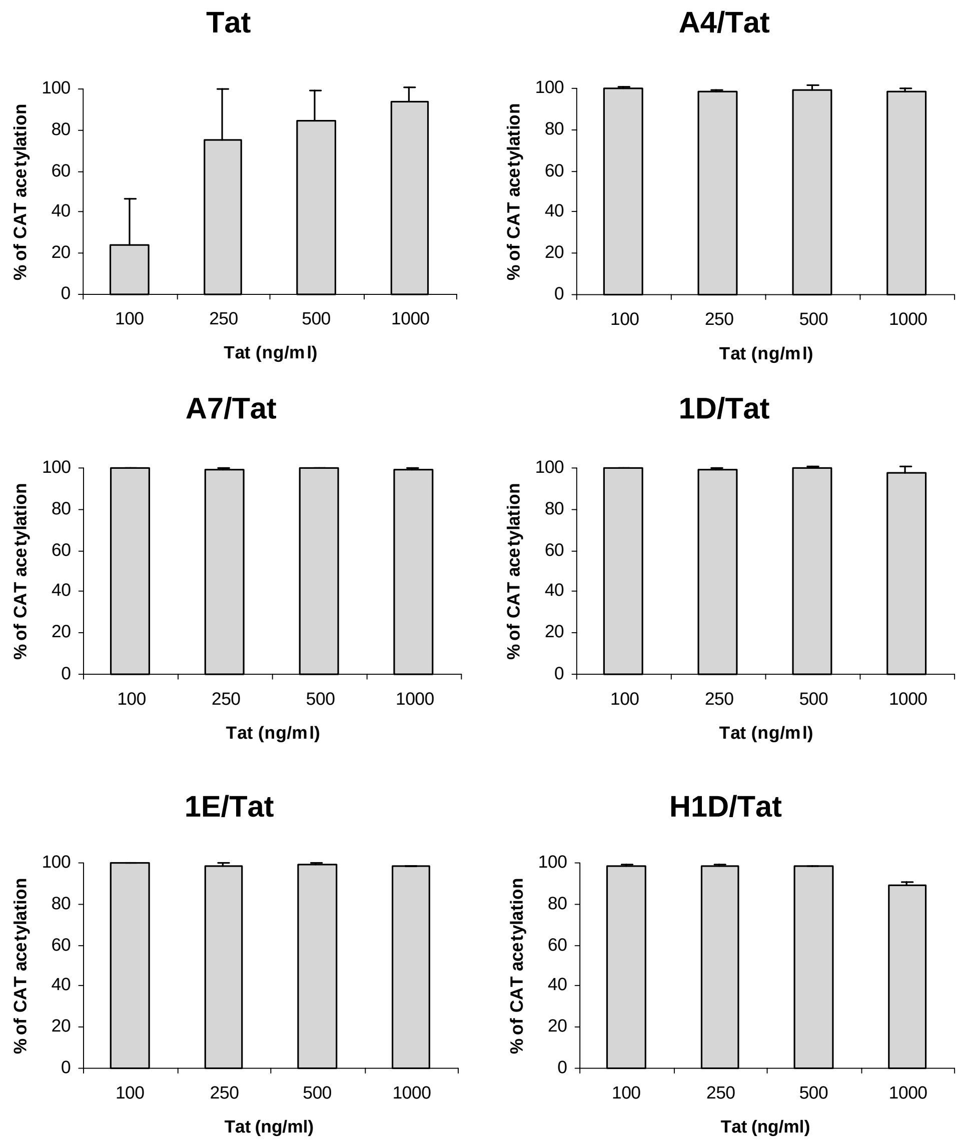

Fig. 9. Analysis of the expression of the HIV-1 Tat protein bound to polymeric microspheres made of poly(styrene) and poly(vinyl alcohol) (A4, A7), and of poly(methyl methacrylate) and Eudragit L100/55 (1D, 1E and H1D), respectively. HL3T1 cells were incubated with increasing amounts of Tat alone, and with the same amounts of Tat bound to each microsphere (30 μg/ml) for 48 h. Results are the mean of three independent experiments (±S.D.).

application as delivery system for vaccine development, samples with the smallest size (1–5 μm range) and diameter dispersion value (0.03–1.02 μm) were selected. For the same reasons, the HIV-1 Tat protein was selected as the model antigen. Due to the presence of a positively charged basic region in the Tat sequence, steric stabilizers with negative charged carboxylate groups (poly(vinyl alcohol) and Eudragit L100/55) were used to produce homogeneous preparations of core-shell microparticles. Polymers and steric stabilizer were chosen based on their biocompatibility [11,44,45]. In addition, poly(methyl methacrylate) and poly(styrene), in the form of nanoparticles, have already been shown to be very attractive as adjuvants in parentally administered vaccines [46–49] and to be slowly biodegradable [50–56]. Moreover, poly(methyl methacrylate) has been used in surgery for over 50 years [11]. Similarly, Eudragit has been approved for human use [57].

The results demonstrate that these novel anionic microparticles can enter several types of professional phagocytic cells and epithelial cells (Figs. 5–7). The polymeric microspheres are phagocyted by murine macrophages at similar efficiency (>80%), and irrespective of their size and chemical composition. Moreover, the microparticles enter monocytes, monocyte-derived dendritic cells, splenocytes and epithelial cells with a high efficiency, ranging from about 12% for murine splenocytes, 18–20% for human monocyte-derived dendritic cells and 25–30% for human monocytes and HeLa cells. Previous studies have shown that the efficiency of delivering molecules to DC using lipofection or electroporation is little or extremely poor [58]. Therefore, the results

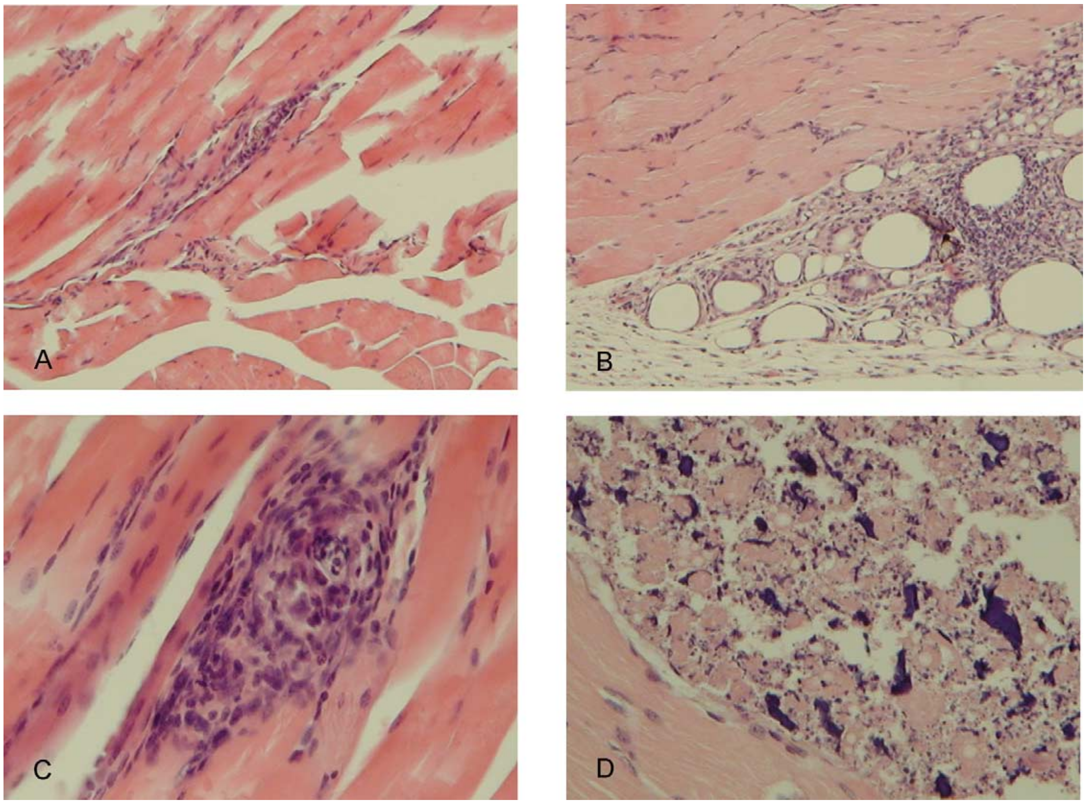

Fig. 10. Histologic examination of the inflammatory reactions present at the site of inoculation. Two representative mice injected i.m. with Tat (2 μg) adsorbed to A7 microparticles (A, C), and Tat (2 μg) in Freund's adjuvant (B, D), at weeks 0, 4, and 8, are shown. A7-Tat inoculation caused a scarce inflammatory reaction (A) in the muscle fibers consisting exclusively of macrophages (C). Tat plus Freund inoculation induced an intense inflammatory reaction prevalently in the adipose tissue surrounding the muscle fibers with presence of macrophages and clear lacunae of lipolysis (B), and in some cases with extensive necrosis constituted by amorphous material and nuclear debris (D). Hematoxylin-eosin staining; A and B: 40×; C: 400×; D: 200×.

suggest that these polymeric particles may function as an efficient delivery systems to APC for generation of effective immune responses in vivo, either by passive transfer or direct immunization.

The results indicate that both classes of microparticles are able to bind Tat on their surface in a dose-dependent fashion (up to 9% (w/w)) (Fig. 3). Tat-adsorption occurs rapidly, being complete in 1 h, it is highly reproducible and the Tat–microsphere complexes are very easy to prepare. The results in cell-free and in tissue culture systems demonstrate that both classes of microparticles bind Tat in its native biological active conformation, and that Tat is gradually released as a bioactive protein into the cells (Figs. 7–9).

Several previous studies described the improvement of vaccines by antigen encapsulation into liposomes [59,60] and biodegradable polymers [61]. However, it has well-established that the encapsulation and release processes expose the antigen to a variety of damaging conditions that often lead to instability and degradation [62]. The advantage of the protein delivery system described here is that both classes of microspheres are designed for adsorption of the antigen at their outer surface. Since microparticles surface can be tailored with positively and negatively charged groups [21,63], ionic interaction with proteins characterized by different isolelectric points can be envisaged. In particular, carboxylated microspheres were designed in order to deliver basic proteins like Tat. This strategy avoids problems of protein instability and/or incomplete release following antigen encapsulation in biodegradable microparticles [64] and seems to be very efficient. This is in agreement with two recent studies describing the use of anionic PLG microparticles for adsorption of the HIV-1 Gag protein at their surface [65,66], and of polymeric lamellar substrate particles for protein adsorption [67].

The observation that microparticles protect Tat from oxidation and consequently from loss of biological activity is noteworthy for their application as protein–vaccine delivery systems, in particular for the development of an anti-HIV vaccine based on Tat and characterized by increased shelf-life in developing countries. Tat is very labile to air, light and temperature and several precautions are needed for the handling and storage of Tat to avoid oxidation. Previous studies have shown that bioactive Tat, but not oxidized Tat, is efficiently taken up by DC at very low doses (in the picomolar range), induces their maturation and increases both allogenic and recall antigen presentation by DC, functioning as both antigen and adjuvant toward Th-1 type immune responses [29,68]. Of note, we and others have also shown that vaccination with native Tat or *tat* DNA protected monkeys against challenge with pathogenic simian-human immunodeficiency virus and that protection correlated with Th-1 responses and CTL activity [22–24,69–71]. This implies that the combination of slow release and depot effect of the Tat–microsphere complexes, together with the preservation of the biological active conformation, may reduce the amount of antigen used in the vaccine and eliminate or reduce the number of booster shots necessary for the success of vaccination [22]. Moreover, Tat delivered by microparticles may be used in prime-boost regimens. In addition, the handling and shelf-life of Tat in vaccine formulations is greatly simplified. These features may be useful for other protein-based vaccines for which immunization with the native bioactive form of the antigen is essential.

Thus, the results indicate that both classes of polymeric microparticles behave in a similar fashion, as concerned protein adsorption and release, maintenance of protein native conformation and biological activity, and the extent of internalization by the cells, irrespective of their chemical composition, surface charge density and size. In addition, microparticles displayed no citotoxicity in vitro, and were safe in vivo. From a manufacturing perspective, this surface-adsorbed antigen delivery system presents several advantages as compared to the antigen entrapment approach, which has lower loading efficiency with loss of 50% or more of the bioactive antigen [62,64]. In addition, the polymeric delivery system described herein can load higher amount of protein (up to 9% (w/w)) as compared to anionic-PLG microparticles (0.5-0.7% (w/w)) used to adsorb HIV−1 p55 Gag protein in the presence of SDS [65]. Finally, both classes of microparticles can be sterilized before adsorption to the sterile antigen, which could simplify and reduce the manufacturing process and costs.

Acknowledgements

This work was supported by grants from the Istituto Superiore di Sanità, from The Italian Concerted Action on HIV-AIDS Vaccine Development (ICAV), from the Associazione Nazionale per la Lotta contro l'AIDS (ANLAIDS), CIB 2002 project and from MURST 60%. We are grateful to E. Fanales, S. Moretti, V. Fiorelli, F. Nappi (Istituto Superiore di Sanità, Roma) for all Tat testing, to C. Celeghini and S. Capitani (University of Ferrara) for assistance at the fluorescent and confocal microscopes, and to L. Magnani (CNR ISOF), R. Voltan, M. Fabris and P.C. Marconi (University of Ferrara) for helpful discussion.

References

[1] O'Hagan DT. Recent advances in vaccine adjuvants for systemic and mucosal administration. J Pharm Pharmacol 1998;59:1–10.

[2] Cahill ES, O'Hagan DT, Illum L, Bernard A, Mills KHJ, Redhead K. Immune responses and protection against *Bordetella pertussis* infection after intranasal immunization of mice with filamentous hemagglutinin in solution or incorporated in biodegradable microparticles. Vaccine 1995;13:455–62.

[3] Singh M, Li XM, Wang H, McGee JP, Zamb T, Koff W, et al. Immunogenicity and protection in small animal models with controlled release tetanus toxoid microparticles as a single-dose vaccine. Infect Immun 1997;5:1716–21.

[4] Singh M, Li XM, Wang H, McGee JP, Zamb T, Koff W, et al. Controlled-release microparticles as a single dose diphteria toxoid vaccine: immunogenicity in small animal models. Vaccine 1998;16:346–52.

[5] Allaoui-Attarki K, Fattal E, Pecquet S, Trolle S, Chachaty E, Couvreur P, et al. Mucosal immunogenicity elicited by oral vaccination with phosphorylcholine encapsulated in poly(DL-lactide-co-glycolide) microspheres. Vaccine 1998;16:685–91.

[6] Aguado MT, Lambert PH. Controlled release vaccines: biodegradable polylactide/polyglycolide (PL/PG) microspheres as antigen vehicles. Immunobiology 1992;184:113–25.

[7] Morris W, Steinhoff MC, Russel PK. Potential of polymer microencapsulation technology for vaccine innovation: review. Vaccine 1994;12:5–11.

[8] Coombes AGA, Lavelle EC, Jenkins PG, Davis SS. Single dose polymeric microparticulate vaccine delivery systems: the influence of formulation conditions on the magnitude and duration of the immune response to a protein antigen. Vaccine 1996;14:1429–38.

[9] Men Y, Thomasin C, Merkle H, Gander B. A single administration of tetanus toxoid in biodegradable microspheres elicit T cell and antibody responses similar or superior to those obtained with aluminium hydroxide. Vaccine 1995;13:683–9.

[10] Jenkins PG, Coombes AGA, Yeh MK, Thomas NW, Davis SS. Aspects of the design and delivery of microparticles for vaccine applications. J Drug Target 1995;3:79–81.

[11] Rihova B. Biocompatibility of biomaterials: hemocompatibility. Adv Drug Deliv Rev 1996;21:157–76.

[12] Anderson JM, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliv Rev 1997;28:5–24.

[13] Nugent J, Wan Po L, Scott E. Design and delivery of non-parental vaccines: review. J Clin Pharm Therap 1998;23:257–85.

[14] Alpar HO, Ward KR, Williamson ED. New strategies in vaccine delivery. STP Pharm Sci 2000;10:269–78.

[15] O'Hagan DT, Rahman D, McGee JP, Jeffery H, Davies MC, Williams P, et al. Biodegradable microparticles as controlled release antigen delivery systems. Immunology 1991;73:239–42.

[16] Eldridge JH, Staas JK, Meulbroek JA, Tice TR, Gilley RM. Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. Infect Immun 1991;59:2978–86.

[17] O'Hagan DT, Jeffery H, Davis SS. Long-term antibody responses in mice following subcutaneous immunization with ovalbumin entrapped in biodegradable microparticles. Vaccine 1993;11:965–9.

[18] Nedrud JG, Lamm ME. Adjuvants and the mucosal immune system. In: Spriggs DR, Koff WC, editors. Topics in vaccine adjuvant research. Boca Raton: CRC Press; 1991. p. 51–67.

[19] O'Hagan DT, Singh M, Gupta RK. Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines. Adv Drug Deliv Rev 1998;32:225–46.

[20] O'Hagan DT. Recent developments in vaccine delivery systems. Curr Drug Target Infect Disord 2001;1:273–86.

[21] Laus M, Sparnacci K, Sante Angeloni A, Valenti S, Tondelli L. Microspheres with hydrophilic and protein-friendly surface as protein delivery systems prepared by dispersion polymerization. J Control Rel 2001;72:280–3.

[22] Cafaro A, Caputo A, Fracasso C, Maggiorella MT, Goletti D, Baroncelli S, et al. Control of SHIV89.6P infection of cynomolgus monkeys by the HIV-1 Tat protein vaccine. Nat Med 1999;5:643–50.

[23] Cafaro A, Caputo A, Maggiorella MT, Baroncelli S, Fracasso C, Pace M, et al. SHIV89.6P pathogenicity in cynomolgus monkeys and control of viral replication and disease onset by human immunodeficiency virus type 1 Tat vaccine. J Med Primatol 2000;29:193–208.
[24] Osterhaus ADME, van Baalen CA, Gruters RA, Schutter M, Siebelink CH, Hulskotte EG, et al. Vaccination with Rev and Tat against AIDS. Vaccine 1999;17:2713–4.
[25] van Baalen CA, Pontesilli O, Huisman RE, Geretti AM, Klein MR, de Wolf F, et al. HIV-1 Rev and Tat specific cytotoxic T lymphocyte frequencies inversely correlates with rapid progression to AIDS. J Gen Virol 1997;78:1913–8.
[26] Arya SK, Guo C, Joseph SF, Wong-Staal F. The trans-activator gene of human T-lymphotropic virus type III (HTLV-III). Science 1985;229:69–73.
[27] Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, et al. Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. J Virol 1993;67:277–87.
[28] Chang HC, Samaniego F, Nair BC, Buonaguro L, Ensoli B. HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. AIDS 1997;11:1421–31.
[29] Fanales-Belasio E, Moretti S, Nappi F, Barillari G, Micheletti F, Cafaro A, et al. Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses. J Immunol 2002;168:197–206.
[30] Caselli E, Betti M, Grossi MP, Balboni PG, Rossi C, Boarini C, et al. DNA immunization with HIV-1 tat mutated in the transactivation domain induces humoral and cellular immune response against wild-type Tat. J Immunol 1999;162:5631–8.
[31] Micheletti F, Monini P, Fortini C, Rimessi P, Bazzaro M, Andreoni M, et al. Identification of cytotoxic T lymphocyte epitopes of human herpesvirus 8. Immunology 2002;106:395–403.
[32] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and citotoxicity assays. J Immunol Methods 1983;65:55–63.
[33] Neri LM, Martelli AM, Maraldi NM. Redistribution of DNA topoisomerase II beta after in vivo stabilization of human erythroleukemic nuclei by heat or Cu^{2+} revealed by confocal microscopy. Microsc Res Tech 1997;36:179–87.
[34] Betti M, Voltan R, Marchisio M, Mantovani I, Boarini C, Nappi F, et al. Characterization of HIV-1 Tat proteins mutated in the transactivation domain for prophylactic and therapeutic application. Vaccine 2001;19:3408–19.
[35] Davis LG, Dibner MD, Battey JF. In: Davis LG, Dibner MD, Battey JF, editors. Basic methods in molecular biology. New York: Elsevier; 1986.
[36] Hsu S-M, Raine L, Fanger H. Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981;29:577–80.
[37] Sokak RR, Rohlf FJ. In: Socak RR, Rohlf FG, editors. Biometry: the principles and practice of statistics in biological research. San Francisco, CA: Freeman; 1981. p. 128–34.
[38] Caldwell KD. Surface modifications with adsorbed poly(ethylene oxide)-based block copolymers poly(ethylene glycol). ACS Symp Ser 1997;680:400–19.
[39] Gref R, Minamitake Y, Peracchia MT, Domb A, Trubetskoy V, Torchilin V, et al. Poly(ethylene glycol)-coated nanospheres: potential carriers for intravenous drug administration. Pharm Biotechnol 1997;10:167–98.
[40] O'Hagan DT. The intestinal uptake of particles and the implications for drug and antigen delivery. J Anat 1996;189:477–82.
[41] Araujo L, Sheppard M, Lobenberg R, Kreuter J. Uptake of PMMA nanoparticles from the gastrointestinal tract after oral administration to rats: modification of the body distribution after suspension in surfactant solutions and in oil vehicles. Intern J Pharmacol 1999;176:209–24.
[42] Zauner W, Farrow NA, Haines AMR. In vitro uptake of poly(styrene) microspheres: effect of particle size, cell line and cell density. J Control Rel 2001;71:39–51.
[43] Ensoli B, Gendelman R, Markham P, Fiorelli V, Colombini S, Raffeld M, et al. Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. Nature 1994;371:674–80.
[44] Fernandez-Urrusuno R, Fattal E, Rodriguez JM, Feger J, Bedossa P, Couvreur P. Effect of polymeric nanoparticle administration on the clearance activity of the mononuclear phagocyte system in mice. J Biomed Mater Res 1996;31:401–8.
[45] Carr KE, Hazzard RA, Reid S, Hodges GM. The effect of size on uptake of orally administered latex microparticles in the small intestine and transport to mesenteric lymph nodes. Pharmacol Res 1996;13:1205–9.
[46] Zimmer A, Kreuter J. Microspheres and nanoparticles used in ocular delivery systems. Adv Drug Deliv Rev 1995;16:61–73.
[47] Zhao Z, Leong KW. Controlled delivery of antigens and adjuvants in vaccine development. J Pharm Sci 1996;85:1261–70.
[48] Kreuter J. Physicochemical characterization of nanoparticles and their potential for vaccine preparation. Vaccine Res 1992;1:93–8.
[49] Stieneker F, Lower J, Kreuter J. Different kinetics of the humoral immune response to inactivated HIV-1 and HIV-2 in mice: modulation by PMMA nanoparticle adjuvant. Vaccine Res 1993;2:111–8.
[50] Kreuter J, Nefzger M, Liehl E, Czok R, Vogels R. Distribution and elimination of poly(methyl methacrylate) nanoparticles after subcutaneous administration to rats. J Pharm Sci 1983;72:1146–9.
[51] Borchard G, Kreuter J. Interaction of serum components with poly(methyl methacrylate) nanoparticles and the resulting body distribution after intravenous injection in rats. J Drug Target 1993;1:15–9.
[52] Maincent P, Thouvenot P, Amicabile C, Hoffman M, Kreutes J, Couvreur P, et al. Lymphatic targeting of polymeric nanoparticles after intraperitoneal administration in rats. Pharmacol Res 1992; 9:1534–9.
[53] Eldridge JH, Hammond CJ, Meulbroek JA, Staas JK, Gilley RM, Tice TR. Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the Peyer's patches. J Control Rel 1990;11:205–14.
[54] Rolland A, Collet B, Le Verge R, Toujas L. Blood clearance and organ distribution of intravenously administered polymethacrylic nanoparticles in mice. J Pharm Sci 1989;78:481–4.
[55] Nefzger M, Kreuter J, Voges R, Liehl E, Czok R. Distribution and elimination of poly(methyl methacrylate) nanoparticles after peroral administration to rats. J Pharm Sci 1984;73:1309–11.
[56] Kreuter J, Tauber U, Illi V. Distribution and elimination of poly(methyl-2-^{14}C-methacrylate) nanoparticle radioactivity after injection in rats and mice. J Pharm Sci 1979;68:1443–7.
[57] Dittgen M, Durrani M, Klehmann K. Acrylic polymers: a review of pharmaceutical applications. STP Pharm Sci 1997;7:403–37.
[58] Arthur J, Butterfield LH, Roth MD, Bui LA, Kiertscher SM, Lau R, et al. A comparison of gene transfer methods in human dendritic cells. Cancer Gene Ther 1997;4:17–25.
[59] Thierry AR, Rabinovich P, Peng B, Mahan LC, Bryant JL, Gallo RC. Characterization of liposome-mediated gene delivery. Expression, stability and pharmacokinetics of plasmid DNA. Gene Ther 1997;4:226–37.
[60] Kersten GFA, Crommelin DJA. Liposomes and ISCOMS as vaccine formulations.. Vaccine 2003;21:915–20.
[61] Johansen P, Men Y, Merkle HP, Gander B. Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination. Eur J Pharm Biopharm 2000;50:129–46.
[62] Hanes J, Cleland JL, Langer R. New advances in microsphere-based single-dose vaccines. Adv Drug Deliv Rev 1997;28:97–119.

[63] Sparnacci K, Laus M, Tondelli L, Magnani L, Bernardi C. Core-shell microspheres by dispersion polymerization as drug delivery systems. Macromol Chem Phys 2002;203:1364–9.
[64] van de Weert M, Hennink WE, Jiskoot W. Protein instability in poly(lactic-co-glycolic acid) microparticles. Pharmacol Res 2000;17:1159–67.
[65] Kazzaz J, Neidleman J, Singh M, Ott G, O'Hagan DT. Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes against human recombinant p55 gag from HIV-1. J Control Rel 2000;67:347–56.
[66] Otten G, Schaefer M, Greer C, Calderon-Cacia M, Coit D, Kazzaz J, et al. Induction of broad and potent anti-human immunodeficiency virus immune responses in rhesus macaques by priming with a DNA vaccine and boosting with protein-adsorbed polylactide coglycolide microparticles. J Virol 2003;77:6087–92.
[67] Jabbal-Gill I, Lin W, Jenkins P, Watts P, Jimenez M, Illun L, et al. Potential of polymeric lamellar substrate particles (PLSP) as adjuvants for vaccines. Vaccine 1999;18:238–50.
[68] Corinti S, Chiarantini L, Dominici S, Laguardia ME, Magnani M, Girolomoni G, et al. Erythrocytes deliver Tat to interferon-γ-treated human dendritic cells for efficient initiation of specific type 1 immune response in vitro. J Leukoc Biol 2002;71:652–8.
[69] Cafaro A, Titti F, Fracasso C, Maggiorella MT, Baroncelli S, Caputo A, et al. Vaccination with DNA containing tat coding sequences and unmethylated CpG motifs protects cynomolgus monkeys upon infection with simian/human immunodeficiency virus (SHIV89.6P). Vaccine 2001;19:2862–77.
[70] Verrier B, Le Grand R, Ataman-Onal Y, Terrat C, Guillon C, Durand PY, et al. Evaluation in rhesus macaques of Tat and Rev-targeted immunization as preventive vaccine against mucosal challenge with SHIV-BX08. DNA Cell Biol 2002;21:653–8.
[71] Agwale SM, Shata MT, Reitz MS, Kalyanaraman VS, Gallo RC, Popovic M, et al. A Tat subunit vaccine confers protective immunity against the immune-modulating activity of the human immunodeficiency virus type-1 Tat protein in mice. Proc Natl Acad Sci USA 2002;99:10037–41.