40 nm, but not 750 or 1,500 nm, Nanoparticles Enter Epidermal CD1a + Cells after Transcutaneous Application on Human Skin

Annika Vogt[1], Behazine Combadiere[2], Sabrina Hadam[1], Karola M. Stieler[1], Juergen Lademann[1], Hans Schaefer[1], Brigitte Autran[2], Wolfram Sterry[1] and Ulrike Blume-Peytavi[1]

Although conventional vaccines have generated major successes in the control of infectious diseases, several obstacles remain in their development against chronic diseases (HIV, tuberculosis), against which no current candidate vaccines yet ensure protection. The transcutaneous route of vaccine administration appears to be a promising approach of targeting vaccines toward antigen-presenting cells (APCs) and thus improving immune responses. We investigated the suitability of nanoparticles in this approach. We found a high density of Langerhans cells (LCs) around hair follicles that, when sorted, readily internalized all size particles. However, flow cytometry after transcutaneous application of 40, 750, or 1,500 nm nanoparticles on human skin samples revealed that only 40 nm particles entered epidermal LC. Fluorescence and laser scan microscopies, which were carried out to identify the penetration pathway of transcutaneously applied nanoparticles, revealed that only 40 nm particles deeply penetrate into vellus hair openings and through the follicular epithelium. We conclude that 40 nm nanoparticles, but not 750 or 1,500 nm nanoparticles, may be efficiently used to transcutaneously deliver vaccine compounds via the hair follicle into cutaneous APCs.

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INTRODUCTION

Vaccines have generated major successes in the control of infectious diseases, but several obstacles remain in the development of vaccines against pandemic chronic diseases, such as HIV or tuberculosis, or in the access to vaccines in developing countries which require safe, well-tolerated, and easy-to-use vaccines. Transcutaneous vaccine delivery offers an advantageous mode of immunization due to the unique ability of cutaneous immune cells, especially Langerhans cells (LCs), to present antigens to the immune system. It is known that strong and efficient immune responses can be induced by targeting vaccines to skin antigen-presenting cells (APCs) (Guebre-Xabier *et al.*, 2003; Partidos *et al.*, 2003). For example, Kenney *et al.* (2004) reported that the intradermal administration of one-fifth of the standard intramuscular dose of an influenza vaccine elicited immunogenicity that was similar to or better than that elicited by conventional intramuscular injection. Recent studies further revealed that vaccination is possible even through intact skin, for example, by patch application (Glenn *et al.*, 2000). Thus, dermal or transdermal delivery of vaccines has provided a great opportunity for further development.

Innovative preparations of vaccines, such as adsorption to nanoparticles, may as well help improve the efficacy of current vaccination strategies. According to Scheicher *et al.* (1995), the uptake of particle-bound antigen by bone marrow-derived dendritic cells triggers their activation and increases their antigen presentation capacity compared to free, that is, not particle-bound, antigen. Shen *et al.* (1997) found in cloned dendritic cells that the presentation of exogenous ovalbumin was markedly enhanced when the antigen was particulate. Similarly, particle-mediated immunization of pigs with plasmid DNA expressing the influenza A hemagglutinin resulted in protection against the following challenge, which was equivalent to or better than a commercial swine influenza vaccine (Macklin *et al.*, 1998). In this study, we investigated the penetration profile of 40, 750, and 1,500 nm nanoparticles on human skin samples by transcutaneous route and their possible targeting to APCs of the skin.

RESULTS

40 nm, but not 750 or 1,500 nm, nanoparticles enter epidermal CD1a + cells after transcutaneous application on human skin

In order to define the cell type accessible to nanoparticles that enter human skin transcutaneously, we carried out

[1]*Department of Dermatology and Allergy, Clinical Research Center for Hair and Skin Physiology, Charite-Universitätsmedizin Berlin, Berlin, Germany and* [2]*Laboratoire d' Immunologie Cellulaire et Tissulaire, Hopital Pitie Salpetriere, Universite Pierre et Marie Curie, Paris, France*

Correspondence: Dr Annika Vogt, Department of Dermatology and Allergy, Clinical Research Center for Hair and Skin Physiology, Charite-Universitätsmedizin Berlin, Berlin, Germany.
E-mail: annika.vogt@charite.de

Abbreviations: APC, antigen-presenting cell; CD, cluster of differentiation; CSSS, cyanacrylate skin surface stripping; LC, Langerhans cell PBS, phosphate-buffered saline

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staining of LCs with anti-cluster of differentiation (CD)1a on human skin samples from healthy volunteers undergoing plastic surgery. We showed a major concentration of CD1a+ cells in the epithelium of a vellus hair follicle infundibulum (Figure 1a). These cells also present LC/dendritic cell morphology, as shown in Figure 1b.

Cyanacrylate skin surface stripping (CSSS) improves the follicular penetration of transcutaneously applied compounds by removing keratinized material, lipids, and other cell debris from the follicular openings, and, occasionally, hair fibers, especially vellus hairs (Mills and Kligman, 1983; Schaefer and Lademann, 2001). One CSSS further removes approx. 30% of the stratum corneum; the remaining stratum corneum and the viable interfollicular epidermis are left intact. After CSSS, 40, 750, or 1,500 nm nanoparticles in aqueous solution were applied on the skin surface, the skin samples were incubated in a humidified chamber at 37°C for 16 hours. LCs were isolated by magnetic cell sorting (MACS) and revealed that only CD1a + cells from skin samples treated with 40 nm nanoparticles, and not 750 nm and 1,500 nm particles, exhibited nanoparticle-associated fluorescence (Figure 2). The percentage of CD1a+particle+ cells was 24.3±15.9% (Figure 2a) compared to control samples. Microscopically, we identified a granular fluorescent staining pattern inside CD1a+ cells, which was highly suggestive for uptake and internalization of the nanoparticles (Figure 2b). In contrast, only 0.4% cells separated from skin samples treated with 750 or 1,500 nm nanoparticles were nanoparticle-positive (Figure 2c and d).

We, thus, showed that LCs, which form a tight meshwork within the epidermis, and which can also be found at high densities around hair follicles, are capable of internalizing 40 nm, but not 750 nm and 1,500 nm, particles when applied transcutaneously.

In vitro isolated LC uptake of 40, 750 and 1,500 nm particles

To verify whether LC suspensions, at all, internalize the nanoparticles, we incubated MACS-separated epidermal LCs for 2.5 hours in the presence of 40, 750, or 1,500 nm nanoparticles. Consistent with previous findings by other groups (Reis e Sousa *et al.*, 1993; Reece *et al.*, 2001), we found a strong increase in cell-associated fluorescence by flow cytometry analysis in all the samples (Figure 3a, d, and g). Fluorescence microscopy of individual cells showed a granular fluorescent staining indicating uptake and internalization not only of 40 nm, but also of 750 and 1,500 nm nanoparticles by LCs (Figure 3b, e, and h).

When experiments were performed at 0°C, cell-associated fluorescence, albeit less intense and not granular, was also found in samples. Microscopic evaluation of these control samples revealed a homogeneous staining pattern confined to the cell borders (Figure 3c) or limited to nanoparticle clusters floating in the cell culture medium, which was highly suggestive for nonspecific adherence of the nanoparticles to the cell surface (Figure 3f and i).

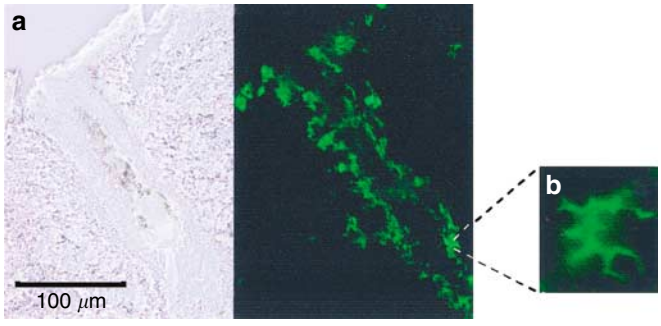

Figure 1. CD1a-positive cells in the epithelium of a human vellus hair follicle infundibulum. Immuofluorescent staining was performed using anti-CD1a-FITC antibody. (**a**) CD1a-positive cells were found at high densities in the epithelium of the human vellus hair follicle infundibulum (original magnification ×20). (**b**) These cells also presented LC morphology (original magnification ×100). (**a**) Bar = 100 μm.

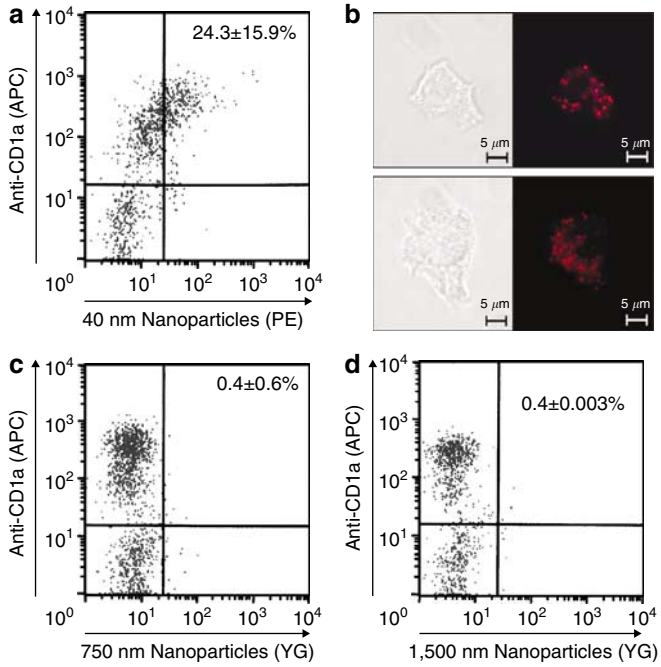

Figure 2. Up-take of 40 nm nanoparticles by epidermal CD1a+ cells after transcutaneous application on human skin. Fluorescent 40 nm (0.1% solids, 2.84×10^{13} particles/ml), 750 nm (0.1% solids, 1.08×10^{10} particles/ml) or 1,500 nm (0.1% solids, 1.35×10^{9} particles/ml) nanoparticles were applied transcutaneously on human skin samples after CSSS. After incubation in a humidified chamber at 37°C for 16 hours, epidermal LCs were separated from the epidermis by MACS separation, stained for CD1a and processed for flow cytometry. In all, 10,000 events were acquired per sample. (**a**) The percentage of cells CD1a+particle+ cells are indicated in the upper right quadrant and increased from 0% in the control samples to 24.3±15.9% in skin samples treated with 40 nm nanoparticles ($n = 6$ different donors). (**b**) These fluorescent cells were also identified by fluorescence microscopy of LC isolates, which showed a granular staining pattern similar to the granular pattern seen after *in vitro* incubation of nanoparticles with isolated LCs (original magnification ×100). The percentage of cells CD1a+particle+ cells separated from skin treated with 750 nm (0.1% solids, 1.08×10^{10} particles/ml) or 1,500 nm (0.1% solids, 1.35×10^{9} particles/ml) nanoparticles are indicated in the upper right quadrant of (**c**, **d**), respectively, and were not significantly different from control samples (750 nm: 0.4±0.6% $n = 3$; 1,500 nm: 0.4±0.003% $n = 3$). (**b**) Bar = 5 μm.

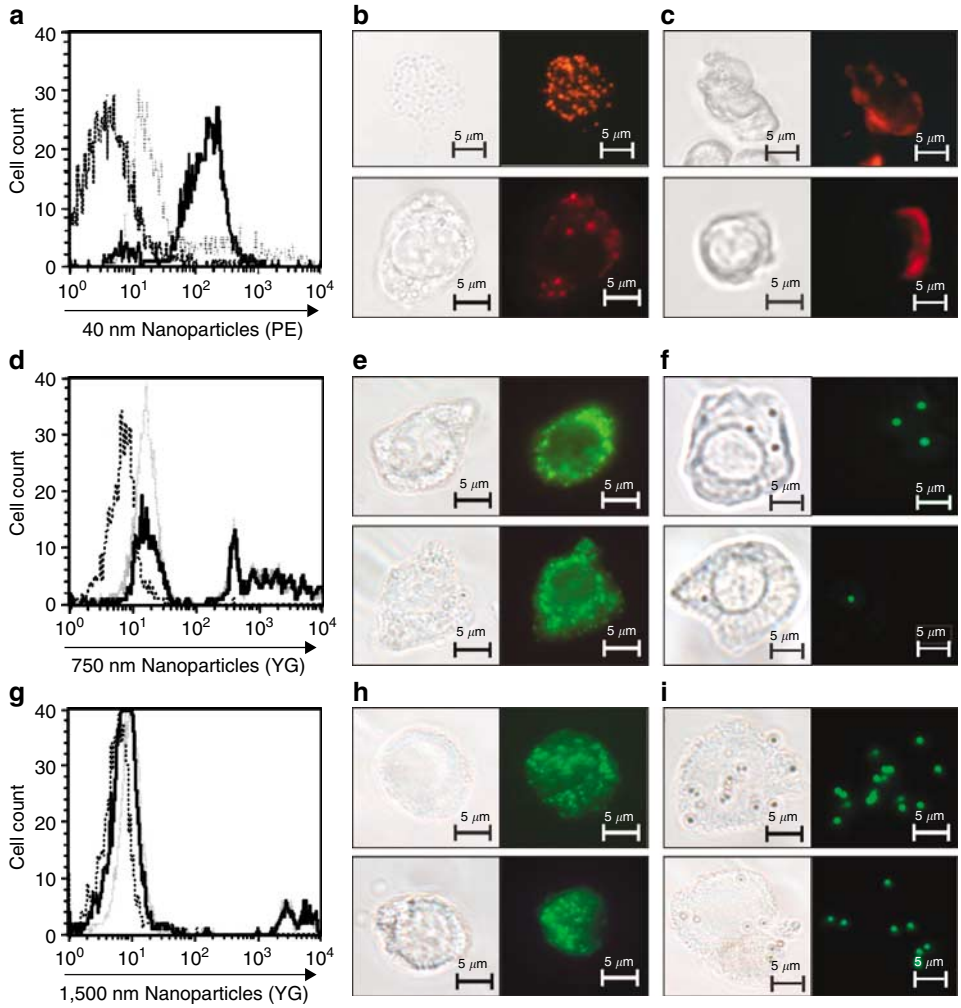

Figure 3. Isolated epidermal LCs in suspension internalize 40, 750, and 1,500 nm nanoparticles. Freshly separated epidermal LCs in culture media were incubated for 2.5 hours at 37°C in the presence of fluorescent 40 nm (0.02% solids, 5.68×10^{10} particles/ml), 750 nm (0.02% solids, 2.16×10^{7} particles/ml), or 1,500 nm (0.02% solids, 2.7×10^{6} particles/ml) nanoparticles, respectively. The experiments were carried out at 37°C and at 0°C as indicated. (**a**, **d**, **g**) Flow cytometry revealed a distinct increase in nanoparticle-related fluorescence in all investigated samples (10,000 events counted) (dotted line: negative control, solid gray line: control at 0°C, solid black line: 37°C). (**b**, **e**, **h**) A granular pattern of fluorescence was observed by fluorescence microscopy on separated cells indicating uptake of nanoparticles by epidermal LCs only at 37°C (original magnification ×100). In contrast, the fluorescence in control samples incubated at 0°C was (**c**) less intense, rather homogeneous and confined to the cell borders or (**f**, **i**) limited to nanoparticle clusters floating in the cell culture medium (original magnification ×100). (**b**, **c**, **e**, **f**, **h**, **i**) Bar = 5 μm.

Penetration pathway of transcutaneously applied 40, 750, and 1,500 nm particles

In order to further elucidate the penetration pathway of transcutaneously applied nanoparticles, we performed fluorescence microscopy on cryosections prepared from skin samples treated with 40 nm (six different donors), 750 nm (three different donors), or 1,500 nm nanoparticles (three different donors), respectively. Each skin sample was split into four blocks of $0.5 \times 0.5\,cm^2$ and cryosections from all blocks were assessed for the presence of vellus hair follicles by fluorescence microscopy. In all, 120 sections from skin samples treated with 40 nm nanoparticles were analyzed, and 60 hair follicle sections were identified. Only the best section of each hair follicle was chosen, subsequent sections of the same hair follicle were excluded from the studies. In all sections investigated by fluorescence microscopy, we found 40 nm nanoparticles penetrating to, at least, the entry level of the sebaceous duct, which, according to our own measurements on 76 vellus hair follicles, corresponds to a depth of $225 \pm 34\,\mu m$ (Figure 4a and b, Vogt *et al.*, in preparation). In all, 26 sections showing complete or almost complete longitudinal sections of vellus hair follicles were analyzed by laser scan microscopy, where we found 40 nm particles penetrating all along the follicular duct, and occasionally 2–3 of 26, also directly into the perifollicular tissue. Fluorescent staining in the dermis was found in 11 out of 26 sections. In contrast, we did not observe nanoparticle-associated fluorescence either below the upper infundibulum or in the dermis, in any of the skin samples treated with 750 or 1,500 nm particles (three different donors each, 60 slides screened, 30

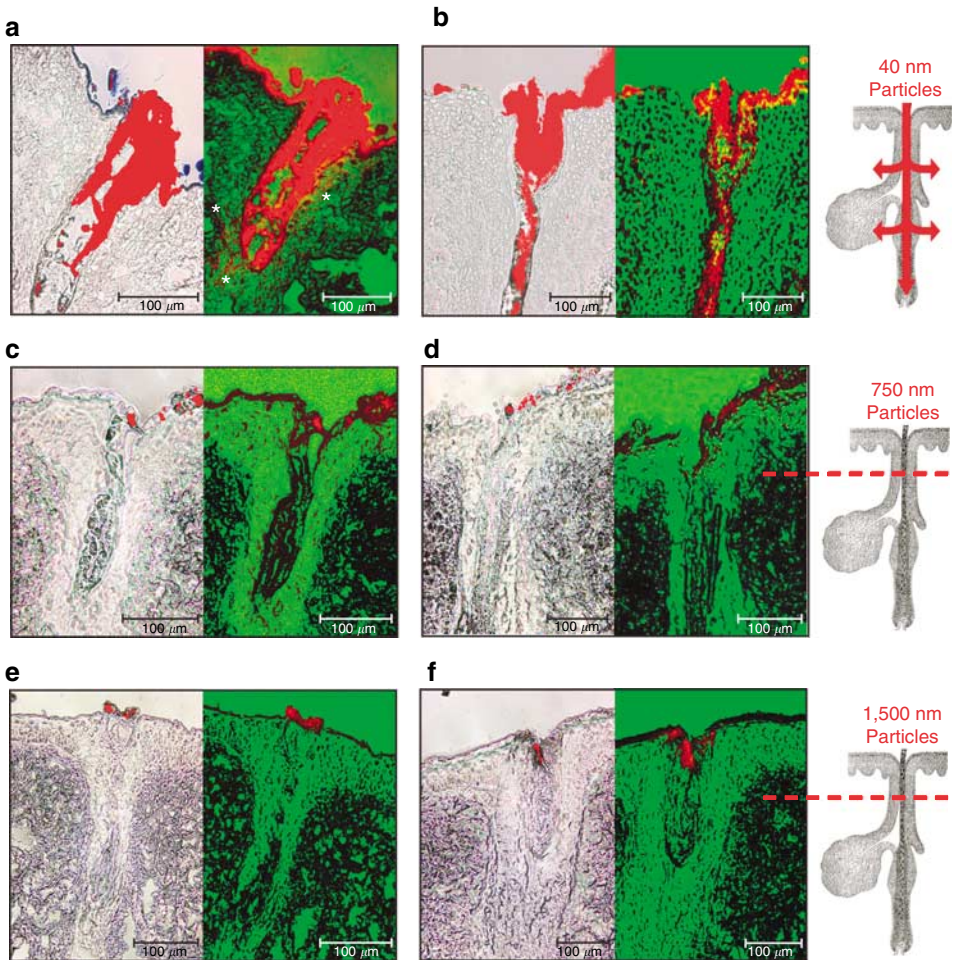

Figure 4. 40 nm, but not 750 or 1,500 nm, nanoparticles penetrate via the vellus hair follicle into the surrounding tissue. Laser scan microscopy were performed on cryosections of skin samples treated with (**a**) 40 nm (0.1% solids, 2.84×10^{13} particles/ml, $n = 6$), (**b**) 750 nm (0.1% solids, 1.08×10^{10} particles/ml), or (**c**) 1,500 nm (0.1% solids, 1.35×10^{9}/ml) nanoparticles. Digital image overlay was used to localize the fluorescent signal on the tissue sections. This figure shows exemplarily corresponding fluorescent microscopy images (black and white native image, red color corresponding to fluorescent signal, original magnification $\times 20$) and laser scan microscopy images of the same hair follicles (black and green native image, red color indicates fluorescent signal, original magnification $\times 20$). (**a**, **b**) We found that 40 nm nanoparticles, in contrast to the larger particles, penetrated deep into vellus hair follicles. (**a**) In addition, laser scan microscopy revealed fluorescent signals in the adjacent tissue (*), indicating that the nanoparticles penetrated into and through the follicular epithelium. Transcutaneously applied (**c**, **d**) 750 nm and (**e**, **f**) 1,500 nm fluorescent nanoparticles, in contrast, aggregated in the infundibulum of human vellus hair follicles. No penetration to deeper parts of the hair follicles and no penetration into viable epidermis was observed in any of the samples. (**a**–**f**) Bar = 100 μm.

sections of hair follicles), although the fluorescence intensity of individual particles of this size is much higher and easier to detect than the fluorescence of 40 nm nanoparticles (Figure 4c–f). Our data confirm that only 40 nm particles penetrated the skin via the follicular route and possibly into the perifollicular dermis, thereby passing the close meshwork of epidermal LCs, which are particularly concentrated in and around the upper parts of human hair follicles as shown in Figure 1 and other studies (Christoph *et al.*, 2000; Taira *et al.*, 2002).

DISCUSSION

In this study, we were able to demonstrate that transcutaneously applied 40 nm nanoparticles, but not 750 or 1,500 nm, penetrated human skin and entered epidermal CD1a+ cells *in vitro*. The interindividual variability of nanoparticle penetration and uptake was high, and most likely a result of factors such as immune status, age of the donor, and modifications related to transport and processing of the skin samples. However, the result of 24.3% nanoparticle-positive cells in skin samples treated with 40 nm nanoparticles compared to 0.4% in skin samples treated with 750 or 1,500 nm nanoparticles demonstrates clearly that only 40 nm nanoparticles were able to cross the skin barrier. In this study, epidermal LCs served as a model cell population to provide first proof of a concept for the use of nanoparticles in transcutaneous vaccination. The immunological role of LCs in transcutaneous vaccination, certainly, needs to be further investigated, as well as the role of dermal APCs. Fluorescence microscopy and laser scan microscopy results suggest that transcutaneously applied 40 nm nanoparticles penetrated predominantly via the follicular route into the perifollicular

dermis, thereby passing the close meshwork of follicular APCs. In contrast, 750 and 1,500 nm nanoparticles aggregated superficially in the hair follicle openings. Consistent with the results obtained by flow cytometry, no deeper penetration was observed and no fluorescent signals in the dermis were observed in these samples. Our results are in accordance with previous findings which showed that nanoparticles, after application on the skin surface, aggregate in follicular openings and penetrate along the follicular duct (Rolland *et al.*, 1993; Mordon *et al.*, 2003). In fact, we recently reported that the penetration depth of transcutaneously applied nanoparticles within the follicular duct of scalp terminal hair follicles depends on the size of the particles, suggesting that nanoparticles allow to selectively target hair follicle compartments such as the entry level of the sebaceous duct or the bulge region (''Follicular Targeting'') (Toll *et al.*, 2004; Vogt *et al.*, 2005). The results presented in this study validate our hypothesis of follicular targeting by differently sized nanoparticles also in vellus hair follicle-bearing skin. Moreover, we were able to demonstrate that, in contrast to larger particles, 40 nm nanoparticles penetrate not only into the hair follicle but also through the epithelium.

LCs can be found at particularly high densities in hair follicle-bearing skin (Thomas *et al.*, 1984). They are particularly concentrated around the upper portion of the hair follicles, where they are found not only in the suprabasal layer but also in the basal layer of the outer root sheath (Christoph *et al.*, 2000; Taira *et al.*, 2002). The loss of the stratum corneum toward the lower part of the hair follicle infundibulum implicates that APCs, which reside in and around the hair follicle, are more accessible for topically applied vaccines than APCs in the interfollicular epidermis, which are shielded by the stratum corneum. Targeting of vaccine to the hair follicle and to follicular APCs may, hence, improve the efficacy of transcutaneous vaccination. CSSS has been shown to open hair follicles for penetration and may represent a valuable tool to enhance the follicular penetration of transcutaneously applied vaccine (Schaefer and Lademann, 2001). Furthermore, hair follicles are not only important entry points but also important reservoirs for topically applied compounds, and CSSS increases this reservoir function of the hair follicle, that is, depots in the follicular duct may help to maintain high local concentrations of vaccine over a prolonged period of time (Lademann *et al.*, 2001). Last but not least, immunostimulatory effects, which have been reported for conventional adhesive tape stripping, may also occur after CSSS of the skin (Katoh *et al.*, 1997; Choi and Maibach, 2003). All these putative effects of CSSS are currently under investigation in our laboratory. In this study, we performed CSSS two times prior to the transcutaneous application of nanoparticles.

From the presented studies, we conclude that only 40 nm particles, but not 750 or 1,500 nm nanoparticles, may be used to transcutaneously deliver active vaccine compounds, via the hair follicle, into cutaneous APCs. Future experimental studies with functional vaccine preparations will further investigate the potential of particle-based vaccines in transcutaneous vaccination.

MATERIAL AND METHODS

Tissue samples

Fresh skin samples (retroauricular region, breast), less than 24 hours after surgical excision, were obtained from healthy volunteers undergoing plastic surgery after informed consent approved by the Institutional Ethics Committee of the Medical Faculty of the Charité-Universitätsmedizin Berlin and according to the ethical rules stated in the Declaration of Helsinki Principles.

Immunofluorescence

For immunofluorescent staining, protein blocking (DAKO protein block) was performed on acetone-fixed cryosections for 1 hour at room temperature. The sections were then incubated for 1 hour with anti-CD1a (1:50, DAKO) in human serum (Biotest, Dreieich, Germany), followed by washing in phosphate-buffered saline (PBS) and incubation with anti-mouse-FITC (1:50, Vector Laboratories, Burlingame, CA) for 45 minutes. After the final washing, the sections were mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) and analyzed by fluorescence microscopy (BX60F3, Olympus, Hamburg, Germany).

Transcutaneous particles application

The skin samples were investigated macroscopically and microscopically for tissue damage. Samples with damaged epidermis were excluded from the study. The application area (1 cm^2 for microscopy studies, 10–16 cm^2 for flow cytometry studies depending on the size and the quality of the skin samples) was delineated by a skin surface marker on the skin samples, leaving safety margins of 0.5 cm to the border of the tissue in order to avoid nonspecific penetration of particles sideways into the tissue. Particles of 1,500 nm, 750 nm (Yellow Green, Fluoresbrite™ Carboxylate Polysciences Inc., Warrington, FL; excitation: 458 nm, emission: 540 nm), and 40 nm (red fluorescent FluoSpheres, Molecular Probes, Eugene, OR; excitation: 580 nm, emission: 605 nm) in PBS, pH 7.4, were sonicated for 3 minutes (Sonorex Super RK102H, Bandelin, Berlin, Germany). Prior to the application of beads, CSSS was performed twice as described elsewhere (Toll *et al.*, 2004) using superglue, UHU GmbH, Buehl/Baden, Germany. Nanoparticles of 20 $\mu l/cm^2$, 40 nm (0.1%, 5.68×10^{10} particles/ml), 750 nm (0.1%, 2.16×10^{7} particles/ml), or 1,500 nm (0.1%, 1.35×10^{6} particles/ml) in PBS, pH 7.4, were applied, respectively. The tissue samples were incubated for 15–16 hours at 37°C in a humidified chamber. For studies on the penetration and the uptake of 40 nm particles, skin samples from six different donors were prepared, and for studies on the penetration and the uptake of 750 and 1,500 nm particles, samples from three different donors were prepared.

MACS separation of epidermal CD1c + cells

Epidermal cell suspensions were generated as described previously (Peiser *et al.*, 2003), with slight modifications. Briefly, dispase digestion (2.4 U/ml Dispase I, Roche, Germany) was performed overnight at 4°C, followed by trypsinization in 0.025% trypsin, 1.5 mM $CaCl_2$ in PBS, pH

7.4, for 10 minutes at 37°C. After resuspension, MACS separation with anti-BDCA-1 (anti-CD1c) antibodies was performed according to the manufacturer's recommenda tion (CD1c blood dendritic cell antigen (BDCA-1) Dendritic Cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany).

Flow cytometry of epidermal LCs after transcutaneous application of particles

MACS-separated epidermal cells after transcutaneous application of particles were stained with anti-CD1a-APC antibody. Skin samples from three different donors were used to evaluate the uptake of transcutaneously applied 750 and 1,500 nm nanoparticles. Skin samples from six different donors were analyzed to assess the uptake of transcutaneously applied nanoparticles and subsequently subjected to flow cytometry using a FACS Calibur (Becton Dickinson, Heidelberg, Germany). In total, 10,000 events in each sample were analyzed using CellQuest® software (Becton Dickinson, Heidelberg, Germany).

Flow cytometry of epidermal LC suspensions incubated in the presence of 40, 750, or 1,500 nm nanoparticles

Nanoparticles of 40 nm (0.02%, 5.68×10^{10} particles/ml), 750 nm (0.02%, 2.16×10^{7} particles/ml), or 1,500 nm (0.02%, 2.7×10^{6} particles/ml) were incubated with 7×10^{5} MACS-separated epidermal LCs in RPMI 1640, 15% fetal calf serum, including penicillin and streptomycin. All samples were incubated for 2.5 hours at 37°C or at 0°C as indicated. Cells were washed in PBS, stained with anti-CD1a-APC antibody, and analyzed by flow cytometry as described above. All experiments were carried out in triplicates.

Fluorescence microscopy and confocal laser scan microscopy

Prior to the freezing of the tissue blocks treated with fluorescent nanoparticles, adhesive tape stripping was performed five times on the skin surface to remove the remaining nanoparticles from the skin surface and to reduce the amount of free nanoparticles on the tissue section. The investigational skin area of 1 cm^2 was excised from the tissue blocks and split into four squares of 0.5×0.5 cm^2. Cryosections of 5 μm (2800 Frigocut-N, Reichert-Jung, Heidelberg, Germany) were prepared from each block. As in previous studies by our group (Toll *et al.*, 2004), cutting was performed with fresh blades from the dermis side towards the epidermis in order to avoid dislocation of nanoparticles from the skin surface on the section. The sections (20 sections per donor, six different donors) were screened by fluorescence microscopy (BX60F3, Olympus, Hamburg, Germany). Sections containing longitudinal sections of vellus hair follicles (26 hair follicles from three different donors in total) were selected for confocal laser scan microscopy analyses (LSM 410 invert, Zeiss, Jena, Germany). Digital image overlay was used to localize the fluorescent signal on the tissue sections.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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