Novel Lipid-Based Hollow-Porous Microparticles as a Platform for Immunoglobulin Delivery to the Respiratory Tract

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Purpose. Delivery of specific antibodies or immunoglobulin constructs to the respiratory tract may be useful for prophylaxis or active treatment of local or systemic disorders. Therefore, we evaluated the utility of lipid-based hollow-porous microparticles (*PulmoSpheres*™) as a potential delivery vehicle for immunoglobulins.

Methods. Lipid-based microparticles loaded with human immunoglobulin (hIgG) or control peptide were synthesized by spray drying and tested for: i) the kinetics of peptide/protein release, using ELISA and bioassays; ii) bioavailability subsequent to nonaqueous liquid instillation into the respiratory tract of BALB/c mice, using ELISA and Western blotting; iii) bioactivity in terms of murine immune response to xenotypic epitopes on human IgG, using ELISA and T cell assays; and iv) mechanisms responsible for the observed enhancement of immune responses, using measurement of antibodies as well as tagged probes.

Results. Human IgG and the control peptide were both readily released from the hollow-porous microspheres once added to an aqueous environment, although the kinetics depended on the compound. Nonaqueous liquid instillation of hIgG formulated in *PulmoSpheres* into the upper and lower respiratory tract of BALB/c mice resulted in systemic biodistribution. The formulated human IgG triggered enhanced local and systemic immune responses against xenotypic epitopes and was associated with receptor-mediated loading of alveolar macrophages.

Conclusions. Formulation of immunoglobulins in hollow-porous microparticles is compatible with local and systemic delivery via the respiratory mucosa and may be used as means to trigger or modulate immune responses.

KEY WORDS: immunoglobulins; hollow-porous particles; immune response; respiratory tract.

INTRODUCTION

Immunoglobulins are of potential value for prophylaxis or treatment of certain diseases. Specific or hyperimmune antibodies raised in various species against microbes or microbial antigens are used for the purpose of passive immunization (1–3). Antibodies directed against cancer-specific antigens may be used to target toxins to tumor cells (4). More recent engineering approaches have extended the utility of immunoglobulins as carriers for bioactive compounds. For example, fusion constructs between immunoglobulin fragments and cytokines, ligands for cellular receptors or antigens, have been used to optimize the bioactivity of such compounds (5–10).

Mucosal delivery of immunoglobulins (either native or engineered) may provide improved efficacy compared to systemic delivery via needle, both for the purpose of active and passive immunization. For example, it is known that only mucosal vaccination can result in secreted IgA responses that are associated with an optimal immunity at the port of entry (11). Finally, the potential exists that chronic or repeated administration of immunoglobulins to the systemic circulation can be achieved by non-invasive administration via the respiratory tract.

In this manuscript, we investigate a new formulation technology for the delivery of bioactive agents, including immunoglobulins, to the respiratory tract. The bioactive agent is formulated into the shell of a novel type of microparticle (*PulmoSpheres*) rather than simply encapsulated. The technology is based on engineering drug-containing hollow-porous microspheres (*PulmoSpheres*™), using a proprietary spray-drying technique. The main excipient is dipalmitoylphosphatidylcholine (DPPC), the principal component of endogenous lung surfactant. *PulmoSpheres* may be delivered to the respiratory tract as dry powders or as suspensions in nonaqueous media including the propellants for metered dose inhalers (e.g., HFA-134a) and long-chain fluorocarbons currently being explored for treating patients suffering from respiratory distress syndrome (e.g., perflubron).

We studied the compatibility of the *PulmoSpheres* technology with formulation, delivery and bioactivity of immunoglobulins, administered to and via the respiratory mucosa. Polyclonal human IgG was chosen as a model immunoglobulin. The presence of xenotypic B and T cell epitopes on human IgG permitted the biologic effects of the formulation to be assesed in BALB/c mice, in terms of local and systemic immunity.

MATERIALS AND METHODS

Animals and Reagents

Female BALB/c mice (4–8 weeks old) and Sprague-Dawley rats were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in specific pathogen-free conditions. Polyclonal human IgG was purchased from Sigma Immunochemical (St. Louis, MO). The HA 110–120 peptide with the sequence SFERFEIFPKE is a known epitope of H1N1 influenza viruses. The peptide was commercially synthesized (Chiron Technologies; Clayton, Australia) and tested for purity by HPLC and mass spectroscopy. The fluorescent derivative of the HA peptide, f-HA peptide, was obtained by covalent coupling carboxyfluorescein to the N-terminus of the peptide (Chiron Technologies).

Preparation of *PulmoSpheres*

Hollow porous hIgG microspheres were prepared by a spray drying technique. An aqueous feed solution was prepared

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ABBREVIATIONS: APC, antigen presenting cells; CFA, complete Freund adjuvant; ELISA, enzyme linked immunosorbent assay; HA, hemagglutinin; hIgG, human IgG; Ig, immunoglobulins; NP, nucleoprotein; DHPE, 1,2 dihexadecanoyl-*sn*-glycero-3-phosphoetanolamine; DPPC, dipalmitoylphosphatidylcholine; MLV, multilamellar vesicles; PFB, perflubron; Pul, *PulmoSpheres*; SUV, single unilamellar vesicles; TcH, T cell hybridoma.

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by mixing two solutions A and B immediately prior to spray-drying. Solution A consisted of a fluorocarbon-in-water emulsion in which 5.2 g of perflubron (Liquivent®, Alliance Pharmaceutical Corp.) was dispersed in 40.3 g of deionized water with the aid of 0.415 g of egg phosphatidylcholine (EPC-100-3, Lipoid, Ludwigschafen, Germany). Fine emulsion droplets (ca. 0.2 μm) were prepared by high-pressure homogenization at 18,000 psi for 5 passes (Avestin Emulsiflex, Ottawa, Canada). Solution B contained 55 mg of human IgG (Sigma Chemicals, St. Louis, MO) and 3.2 mg of hydroxyethylstarch (HES, Ajinomoto, Japan) dissolved in 2 g of normal saline. One eighth of solution A by volume was added to solution B. The combined feed solution was spray-dried with a B-191 Mini Spray-Drier (Büchi, Flawil, Switzerland) under the following conditions: aspiration: 100%, inlet temperature: 85°C; outlet temperature: 61°C; feed pump: 2.5 ml min^{-1} N_2 flow: 2800 L/hr. A 150-mesh stainless steel sieving screen was placed over the cyclone exit opening to aid with the recovery of powder. The powder was collected from the cyclone, sieving screen, and collection jar using perflubron as a recovery medium. The hIgG *PulmoSpheres* suspension in perflubron was subsequently frozen at −60°C and lyophilized. A free flowing white powder was obtained. The hollow porous IgG particles had a volume-weighted mean aerodynamic diameter (VMAD) of 4.575 ± 1.89 μm as determined by a time-of-flight analytical method (Aerosizer, Amherst Process Instruments, Amherst, MA). That was associated with geometric diameters around 7 μm (7.0 ± 2.9) as determined by laser difraction and bulk density of 0.4 g/cubic centimeter (tap method, Van Kel Industries Inc., Edison, NJ). Using scanning and transmission electron microscopy, the *PulmoSpheres* exhibited a thin-walled porous morphology, with pore diameter on the order of 50–300 nm.

The f-HA 110–120 and NP 147–155 peptide microspheres were prepared in the same fashion. In this case, solution B consisted of 18 mg of HA 110–120 or NP 147–155 peptide and 1 mg of hydroxyethyl starch dissolved in 5 g of deionized water.

Fluorescent-labeled microspheres were prepared by dissolving 2 mg Texas-red DHPE (Molecular Probes, Eugene, OR) and 1.3 g E100-3 in chloroform:methanol (2:1, v/v). The chloroform/methanol was then removed using a Buchi Roto Vap. The E100-3/Texas Red DHPE thin film was then dispersed into 70 ml hot deionized water (60 to 70°C). Thirty grams of perflubron was then emulsified in the solution as described above. Solution B consisted of 120 mg HES dissolved in 25 ml deionized water. Both solutions were combined and spray-dried as previously described. A free flowing fuchsia-colored powder was obtained. The fluorescent-labeled microspheres had a VMAD of 1.032 ± 1.437 μm.

Small unilamellar vesicles (SUV's) and multilamellar vesicles (MLV's) were prepared by preparing a thin film of 100 mg E100-3 as described above. In each case, 20 mL of Dulbecco phosphate buffered saline (Sigma) was added. The MLV's were prepared by sonicating the aqueous dispersion of E-100 for 30 min using a Branson sonication bath (Fisher Scientific, Pittsburgh, PA). The SUV's were first sonicated like the MLV's, followed by high pressure homogenization at 18,000 psi for 5 discrete passes using the Avestin C-5 emulsiflex. Particle size analysis was performed via laser light scattering (Horiba LA700, Irvine, CA). The median particle diameter of the MLV's and SUV's was determined to be 8.5 ± 2.1 μm and 0.11 ± 0.03 μm, respectively.

Administration of Formulations to BALB/c Mice and Sampling

PulmoSpheres were suspended in perflubron at a concentration of 5 mg/ml. When the particles are placed in a non-aqueous medium, the medium permeates within the particles creating a novel form of suspension—termed *homodispersion*—where the continuous and dispersed phases are identical, separated by a solid interfacial layer of drug and excipient (phosphatidylcholine and lactose or hydroxyethylstarch as well as minor excipients like Texas Red-DSPC and calcium chloride). That is suggested by the physical characteristics of the suspension, namely low sedimentation or creaming rate. The particles remain stable in nonaqueous environment as assessed by fluorescent microscopy.

Various volumes of this suspension (20–70 μl) were then administered to anesthetized BALB/c mice. In the case of nasal administration, mice anesthetized with metofane received the suspension in a dropwise manner using P20 pipets. In the case of intratracheal administration, mice previously anesthetized with ketamine (200 mg/kg) + xylazine (10 mg/kg), were surgically prepared for intratracheal instillation. Briefly, the skin was incised in the cervical region followed by blunt dissection in order to expose the trachea below the laringeal cartilage. Administration was accomplished by instillation into the trachea using a syringe with 27-gauge needle bent at 90°. The incisions were sutured and the mice were allowed to recover. Control mice were treated with various formulations administered via intratracheal, intranasal, intravenous or intraperitoneal routes.

The blood sampling was carried out by retro-orbital or cardiac puncture. Serum was separated using Microtainer serum separator tubes (Becton Dickinson, Franklin Lakes, NJ). In some cases, spleens and lungs were harvested for preparation of single cell suspensions. For this, spleens were simply homogenized and passed through cell strainers (Becton Dickinson; Franklin Lakes, NW). Lungs were first digested with collagenase from *C. histolyticum* (Sigma) (1 mg/15 ml of DMEM-1% BSA) for 90 minutes at 37°C (12,13). Cells were counted using an automated System 9000 cell counter (Seron-Baker Diagnostics; Allentown, PA).

Detection and Measurement of Immunoglobulins by ELISA and Western Blotting

The concentration of hIgG was measured by capture or direct ELISA. Microwell plates (Nunc-Immunoplate, Nunc; Roskilde, Denmark) were coated overnight at 4°C with mouse anti-human kappa light chain monoclonal antibodies (Sigma, cat. no. K-4377) in carbonate buffer (1:500, pH = 9.6) and blocked with PBS-15% mouse serum for at least 1 hour at room temperature. The wells were washed and incubated with various dilutions of samples for 2 hours at room temperature. As negative controls, we included various dilutions of mouse serum. After extensive washing, the wells were incubated for 1 hour at room temperature with mouse anti-human IgG monoclonal antibody conjugated with alkaline phosphatase (Sigma, cat. no. A-2064) diluted at 1:1000 in PBS supplemented with 15% mouse serum and 0.05% Tween 20 (Sigma). The assay was developed using soluble substrate (pNPP, Sigma Immunochemicals) and the results were read using an automatic plate reader at an absorbance of 405 nm (ThermoMax, Molecular Devices; Menlo Park, CA). Direct ELISA was carried out after coating

the wells with the samples and using the same anti-human IgG reagent coupled with alkaline phosphatase. As controls, normal saline and samples harvested from naïve mice were used. In addition, standard curves were constructed using known concentrations of hIgG. The interpolation of the results was carried out using SoftMax software (Molecular Devices).

SDS PAGE electrophoresis was performed under non-reducing conditions and followed by transfer to nitrocellulose (Baxter Scientific; McGaw Park, IL). After overnight blocking with 1% BSA at 4°C, mouse anti-human IgG coupled with HRP (Southern Biotechnology Associates, Birmingham, AL) was added at a concentration of 1:60,000 in 1% BSA for 2 hours. The assay was developed using ECL detection kit for HRP from Amersham (Arlington Heights, IL).

Measurement of Humoral and Cellular Immune Responses

The measurement of antibody titers against hIgG was carried out from serum samples and bronchoalveolar washes that were harvested as described elsewhere (14). Wells were coated with 10 μg/ml of hIgG, blocked with 15% goat serum and incubated for two hours with various dilutions of samples. After extensive washing, goat anti-mouse IgG (Fc specific) antibodies conjugated with alkaline phosphatase (Sigma, cat. no. A7434) were incubated for 1 hour at a dilution of 1:1000 in PBS-15% goat with 0.05% Tween-20. Alternatively, we have used monoclonal rat anti-mouse IgG2a or IgG1 antibodies coupled with biotin and a supplementary step consisting of streptavidin-alkaline phosphatase conjugate (Biosource International; Camarillo, CA). The binding to BSA was subtracted from the binding to hIgG and titers were calculated based on endpoint dilutions that gave a signal at least three times higher than that of samples from naïve mice.

For T cell activity, 2×10^5 responder cells/0.5 ml/well were incubated with the same number of haplotype-matched mitomycin-treated splenocytes for 72 hours, in the presence of 10 μg/ml of hIgG. The cell culture medium consisted of RPMI supplemented with 10% FCS. The concentration of IFN-γ, IL-4 and IL-2 in supernatants was determined by ELISA using reagents from Biosource International. As a negative control, we have used cell culture supernatants resulting from incubation in the absence of hIgG. *In vitro* activity of the f-HA peptide was determined using a T cell activation assay described elsewhere (15).

In Vitro Characterization of PulmoSpheres and Determination of Release Kinetics of hIgG and HA Peptide

Precise measurement of the concentration of hIgG in the *PulmoSpheres* was carried out by capture ELISA, using reagents specific for a k light chain epitope and the heavy chain, thus verifying the heteromeric molecular structure of IgG. *PulmoSpheres* loaded with hIgG were suspended for 24 hours in sterile PBS at a powder concentration of 5 mg/ml. Known concentrations of hIgG in saline and a saline solution of hIgG supplemented with *PulmoSpheres* loaded with an unrelated compound (peptide NP 147-155 of influenza virus) were used as controls. The former was used to generate a standard calibration curve, the latter was included to demonstrate the lack of interference of excipients on the ELISA assay. The results were confirmed by direct ELISA using anti-heavy chain reagent.

To assess the kinetics of release of hIgG or f-HA peptide, loaded *PulmoSpheres* were placed in one compartment of dissociation chamber, which is separated from the sampling compartment by a membrane with <0.4 μm pores (Corning Costar; Cambridge, MA). The integrity of the membranes was checked with *PulmoSpheres* covalently labeled with Texas Red: at no time were the tagged *PulmoSpheres* ever observed in the sampling chamber. Thus any protein appearing in the sampling side reflects dissolved protein. Dry *PulmoSpheres* were exposed to normal saline at time zero. The dissociation chambers were placed at 37°C on a horizontal shaker (frequency of 60 cpm) and small volumes were sampled from the second compartment at various intervals. The concentration of hIgG was measured by capture ELISA and the concentration of f-HA peptide by T cell assay, as described above.

Assessment of Loading of Bronchoalveolar (BAL) Macrophages with *PulmoSpheres*

Bronchoalveolar macrophages were harvested from anesthetized Sprague-Dawley rats by gently washing the airways with 3 ml of PBS twice via a tracheal catheter, using previously described method (16, 17). The presence of functional macrophages was assessed by pulsing with 1 μm polystyrene beads labeled with fluoresceine (Molecular probes, Eugene, OR); approximately 30% of lavaged cells incorporated more than 5 beads/cell. For the assessment of *PulmoSphere* uptake, the cells were stored on ice, washed with cold RPMI, counted and resuspended in RPMI supplemented with 2% serum-free aminoacid solution (MaxiMab; J. Brooks Labs, San Diego-CA). Subsequently, the cells were incubated at a concentration of 10^5/100 μl of medium in wells of 96-well flat bottom plates (Becton Dickinson; Franklin Lakes, NJ) containing dry PulmoSpheres (100 μg/well). The PulmoSpheres used in these experiments contained lipid covalently tagged with Texas Red. The plates were incubated at 37°C for 1 hour in the following manner: 30 minutes on horizontal shaker (60 cycles/min) and 30 minutes still. The supernatant and non-adherent cells were subsequently removed. The adherent cells were washed in the wells three times using normal saline and their nuclei were stained with SYTO-16 (Molecular Probes; Eugene, OR) according to manufacturer's instructions, for 30 minutes at room temperature. After another wash with PBS, the cells were visualized using fluorescent microscopy (Sedival, Jena) with filters for Texas Red (red) and SYTO-16 (green). The percentage of cells (corresponding to SYTO-16^+ events) exhibiting internalization of Texas Red associated with *PulmoSpheres*, was estimated by microscopy. More than 20 fields corresponding to 500 cells/well were randomly assessed.

The PulmoSpheres used in the dose-effect experiment contained various concentrations of hIgG (from 0 to 50%). In a separate procedure, 100 μg of *PulmoSpheres* loaded with 50% of hIgG were incubated with macrophages in the manner described above, in the presence of various amounts of soluble hIgG (50, 150 and 500 μg respectively), in order to test the competitive inhibition by soluble ligand on the uptake of *PulmoSpheres*.

Finally, in order to assess the co-delivery of hIgG and *PulmoSpheres*, we constructed particles loaded with hIgG that were previously tagged with Oregon Green according to the manufacturer's protocol (Molecular Probes, Eugene, OR). The particles contained 25% tagged hIgG and 0.5% lipid (DPPC) tagged with Texas Red. The macrophages were pulsed with such particles and processed for fluorescent microscopy as described above. For *in vivo* studies, we used anesthetized and tracheally-intubated Sprague Dawley rats insufflated (*Insufflator*, Penn-Century™, Philadelphia, Pennsylvania) with 1 mg of tagged *PulmoSpheres* loaded with Oregon Green-IgG. After one hour, the broncholaveolar lavage cells were harvested from three animals, pooled, washed with 4°C-cold medium and run on a flowcytometer (Epics-Profile II, Coulter; Santa Ana, CA). The acquisition of data was carried out in a bicolor mode (green/red for Oregon Green and Texas Red, respectively). As reference, we have used cells harvested from rats treated by liquid instillation or liquid aerosolization (*Insufflator*) with matched amounts of Oregon Green-hIgG (250 μg/rat) in 100 μl of saline.

RESULTS

Release of hIgG and f-HA Peptide from *PulmoSpheres*

We studied the kinetics of release of hIgG from *PulmoSpheres* (20% hIgG) in an aqueous environment. *PulmoSpheres* are stable either as dry powder or when resuspended in non-aqueous propellants like perfluorocarbons or HFA. Their exposure to saline mimics to a certain extent the *in vivo* conditions encountered by the *PulmoSpheres* upon delivery to the respiratory tract. As controls, *PulmoSpheres* loaded with an unrelated peptide (f-HA) and lyophilized IgG or f-HA peptide were used. All formulations were run in parallel. The concentration of hIgG was measured by capture ELISA using known standards and that of f-HA peptide by a T cell bioassay. As shown in Fig. 1A, approximately 80% of hIgG was released from *PulmoSpheres* within 6 hours. Complete release required overnight exposure to saline. This response was significantly different compared to the rapid kinetics observed with lyophilized hIgG exposed to saline or compared to the fast release of f-HA (Fig. 1). In the case of formulated or non-formulated f-HA peptide, essentially all the peptide went into solution within 1 hour. This result was confirmed by semiquantitative fluorescence microscopy using Texas Red labeled microspheres and fluorescein tagged peptide (f-HA) (data not shown). Whereas 50% release of f-HA required 20 minutes, 50% release of hIgG required 60 minutes. Thus, the profile of release from *PulmoSpheres* depends on the compound formulated within the microspheres.

Delivery of hIgG Loaded *PulmoSpheres* to the Respiratory Tract of BALB/c Mice: Systemic Bioavailability

PulmoSpheres loaded with hIgG (20%) were resuspended in perflubron to a concentration of 5 mg/ml, which corresponds to 1 mg/ml of hIgG. The suspension was administered to BALB/c mice via nasal or tracheal route. Capture ELISA on blood samples demonstrated that both intranasal and intratracheal inoculation of hIgG-loaded *PulmoSpheres* suspended in perflubron, resulted in significant and persistent levels of hIgG being

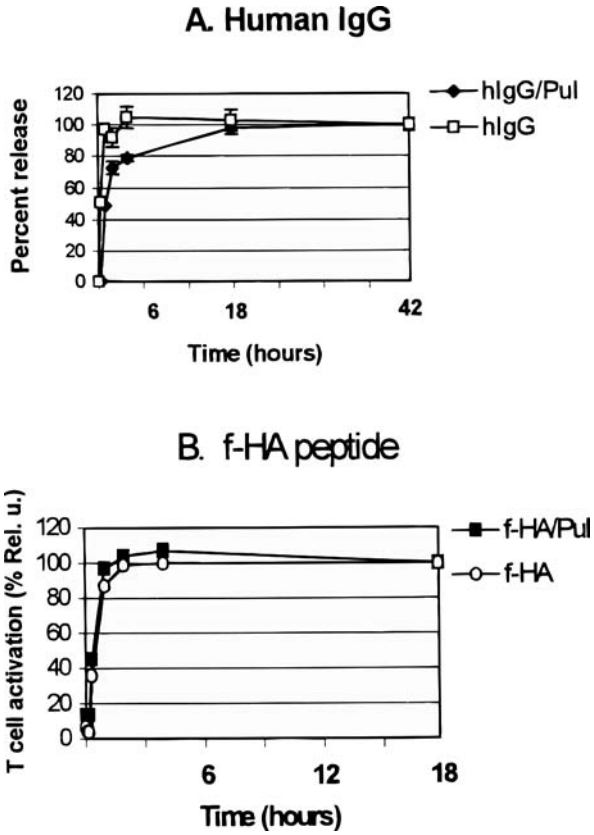

Fig. 1. Release of antigens from *PulmoSpheres* in aqueous environment. (A) Release kinetics of human IgG from *PulmoSpheres* (hIgG/Pul) versus lyophilizate (hIgG). (B) Release kinetics of f-HA peptide from PulmoSpheres (f-HA/Pul) versus lyophilizate (f-HA). Data were expressed as percentage of maximum signal determined by capture ELISA in the case of hIgG and T activation assay in the case of f-HA peptide. The experiment was run in duplicates and data are shown as means ± SE.

delivered to the systemic circulation (Fig. 2A,B). The time to reach peak values was on the order of 24–48 hours and the circulating antibodies lasted at least 14 days. Compared to intravenous administration, the bioavailabilities for hIgG (molecular weight around 150 kDa) were approximately 1.5% in the case of intranasal delivery versus 27% for intratracheal instillation. Furthermore, Western blot analysis of sera from mice that received hIgG via the respiratory tract strongly suggested that translocated immunoglobulins remained structurally intact, since no antigenic fragments could be revealed in a soluble form (Fig. 2C). The systemic bioavailability by intratracheal instillation of hIgG in saline was slightly but reproducibly higher as compared to that obtained in the case of *PulmoSpheres* (range of 32–40% versus 25–27%). Thus, hIgG formulation in *PulmoSpheres* can be successfully delivered via the respiratory tract by liquid instillation.

Delivery of hIgG Loaded *PulmoSpheres* to the Respiratory Tract of BALB/c Mice: Immune Response to Xenotypic Epitopes

We took advantage of the presence of xenotypic B and T cell epitopes on hIgG in order to study the local and systemic

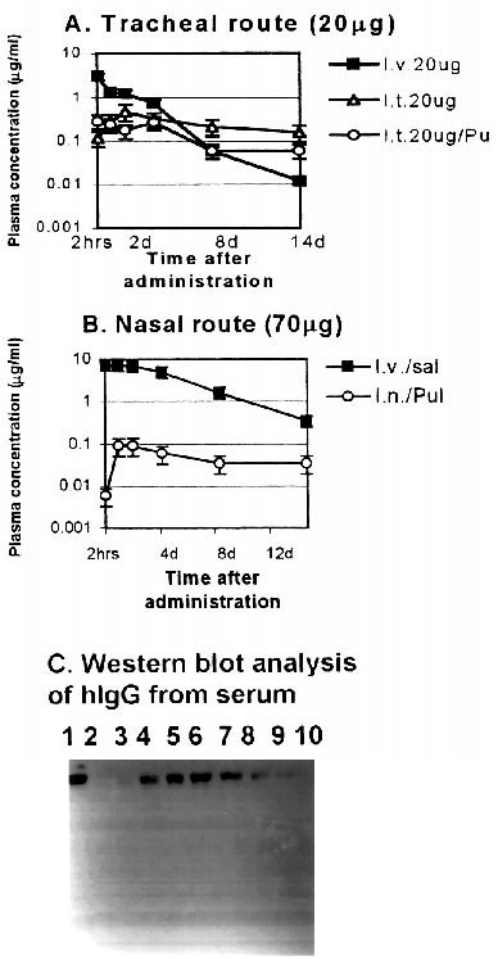

Fig. 2. *In vivo* delivery of hIgG formulated in *PulmoSpheres*, via liquid instillation into the respiratory tract of BALB/c mice: kinetics of serum concentration measured by ELISA. (A) Serum concentration subsequent to intratracheal (i.t.) delivery of *PulmoSpheres* in perflubron versus i.t. delivery in saline or intravenous (i.v.) delivery of dose matched hIgG (20 μg) in saline (n = 4). (B) Serum concentration subsequent to nasal delivery of *PulmoSpheres* in perflubron (i.n./Pul) versus intravenous delivery of dose matched hIgG (70 μg) in saline (i.v./sal). For panels (A) and (B), results are shown as mean ± SEM; n = 4 mice/group. The bioavailabilities were calculated as the ratio of areas under the curve (intranasal or intratracheal/intravenous) using specific software. (C) Integrity of human IgG in the serum of BALB/c mice after delivery via the respiratory tract, assessed by Western blotting. Line 1, hIgG positive control; line 2, mouse IgG—negative control. Lines 3–10, serum samples at 2 hours, 1, 2, 4, 8, 10 and 14 days after administration (data representative for three mice).

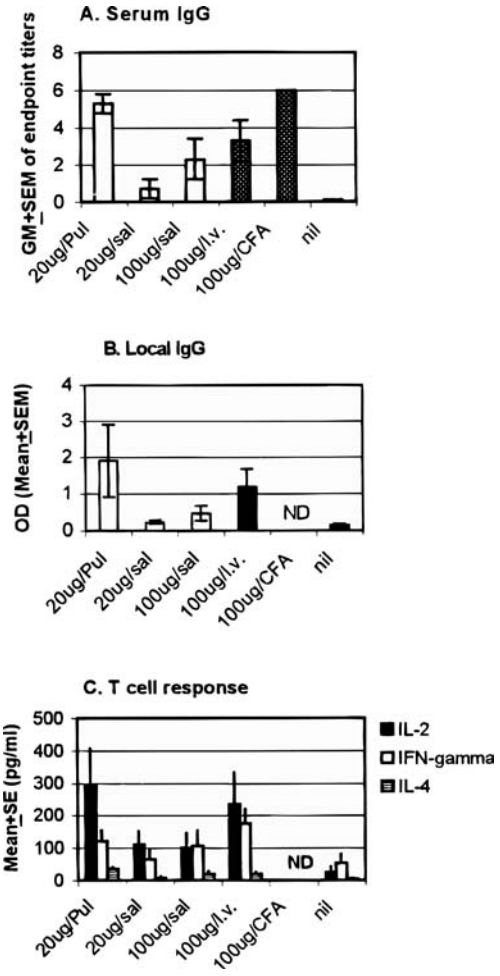

Fig. 3. The immune response against hIgG formulated in *PulmoSpheres* and delivered via the tracheal route. (A) Specific IgG response in the sera against hIgG at two weeks after immunization via the respiratory tract (open bars) or by injection (closed bars). (B) Titers of specific IgG in the bronchoalveolar lavage of mice treated with hIgG via the respiratory tract (open bars) or by injection (closed bars). (C) Specific T cell response representing the cytokine production by splenocytes in vitro stimulated with hIgG. The results are expressed as means ± SE of 3 animals/group.

immune responses to immunoglobulins formulated in *PulmoSpheres*. This may be of predictive value for the bioactivity of Ig constructs bearing immunogenic epitopes. Administration of hIgG loaded *PulmoSpheres* (20% hIgG) via liquid instillation in perflubron into the trachea of BALB/c mice resulted in generation of high titers of specific IgG antibodies in the serum as well as bronchoalveolar lavage fluid (Fig. 3A,B). This was paralleled by enhanced production of IL-2, IFN-γ and IL-4 by *in vitro* stimulated splenocytes (Fig. 3C). Significantly lower immune responses were noted in the case of mice inoculated with a matched dose of hIgG formulated in saline. Even administration of higher amounts of hIgG in saline, via the respiratory tract or parenteral route, did not restore the magnitude of immunity seen with IgG formulated in *PulmoSpheres*. In fact, such enhanced immunity was noted only in the case of subcutaneously administered hIgG formulated in complete Freund's adjuvant (CFA), a strong adjuvant restricted primarily to animal use (Fig. 3A).

The results were confirmed using intranasal inoculation (Fig. 4). Formulated hIgG elicited systemic humoral responses that were faster, of higher magnitude with lower intersubject variability as compared to the response of mice instilled with similar doses of saline-formulated hIgG. The assessment of isotype profiles of specific antibodies by ELISA using γ1 and γ2a-specific reagents, showed enhanced responses for both IgG1 and IgG2a (Fig. 4B,C). Thus, formulation of hIgG in

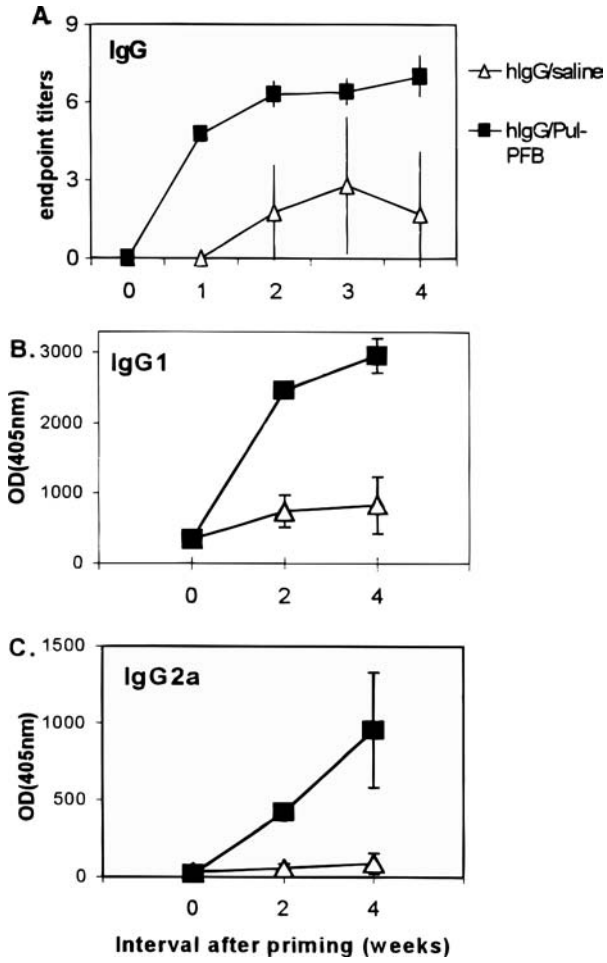

Fig. 4. The humoral immune response against hIgG formulated in *PulmoSpheres* subsequent to nasal administration. The titers of specific IgG antibodies were measured in serum (A) and the results were expressed as means ± SE of $\log_2$ [endpoint titers/100]. The presence of IgG1 (B) and IgG2a (C) antibodies against hIgG was assessed in serum and the results were expressed as average absorption ± SE at 1:200 dilution (n = 4 mice/group).

PulmoSpheres is associated with enhanced immunity to xenotypic epitopes following delivery into the respiratory tract of BALB/c mice.

Mechanisms Responsible for the Enhanced Immunity Against Formulated hIgG

At least two mechanisms may explain the enhanced immunity in response to hIgG formulated in *PulmoSpheres*: a potential intrinsic adjuvant effect of the excipients, or an effect of the formulation on the antigen itself (aggregation, slow release or targeting to antigen presenting cells).

In order to discriminate between these two hypotheses, we administered hIgG-loaded *PulmoSpheres* to BALB/c mice via intraperitoneal inoculation and compared the effects of the following controls: hIgG in saline and hIgG co-administered with either empty *PulmoSpheres* shells (just excipients), small unilamellar vesicles (SUVs), or multilamellar vesicles (MLVs). The IgG response at 7 and 14 days after inoculation were measured (Fig. 5). It was evident that addition of hIgG in saline to empty *PulmoSpheres*, SUVs, or MLVs could not generate the enhanced immunity of hIgG formulated in *PulmoSpheres*. The antibody titers at 7 days after inoculation were consistent with the results obtained at 14 days for each group. In a control experiment, hIgG-loaded *PulmoSpheres* were innoculated within 10 minutes after being resuspended in perflubron or saline. No difference in the magnitude of immunity was seen (data not shown). These data suggest that incorporation of hIgG into *PulmoSpheres* was responsible for the enhanced immunity, rather than any intrinsic adjuvant activity of the *PulmoSphere* excipients or the perflubron carrier.

To further understand the mechanism of enhanced immune activity of formulated hIgG, we assessed the ability of hIgG to facilitate the uptake of *PulmoSpheres* by macrophages, that are well known antigen presenting cells. Incubation of hIgG-loaded but not unloaded *PulmoSpheres* with bronchoalveolar macrophages, was followed by significant cellular uptake (Fig. 6A). The phagocytosis of hIgG-*PulmoSpheres* was highly dependent on the content of hIgG (Fig. 6A). Addition of soluble hIgG up to 1.5 mg/ml to empty *PulmoSpheres* did not facilitate their uptake (less than 0.1% Texas Red^+ cells). Further, the

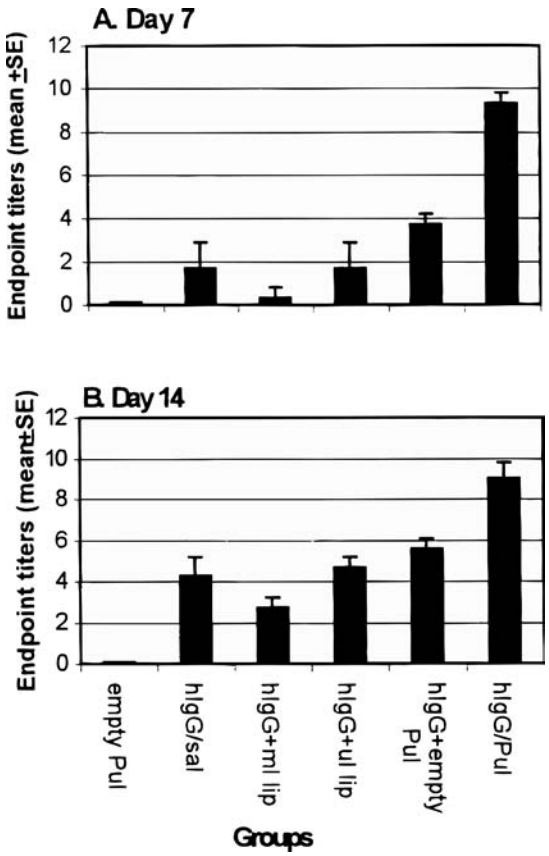

Fig. 5. Effect of formulation on the immune response of BALB/c mice to hIgG. The serum titers of IgG antibodies at day 7 (A) and 14 (B) after intraperitoneal immunization are expressed as means ± SE of $\log_2$ [endpoint titers/100] (n = 3). Mice were injected with matched doses (100 μg) of hIgG in saline added to empty *PulmoSpheres* (hIgG + empty Pul), MLV (ml/lip) or SUV (ul/lip) composed of DPPC. Alternatively the mice were immunized with hIgG formulated in *PulmoSpheres* (hIgG/Pul) or dissolved in saline.

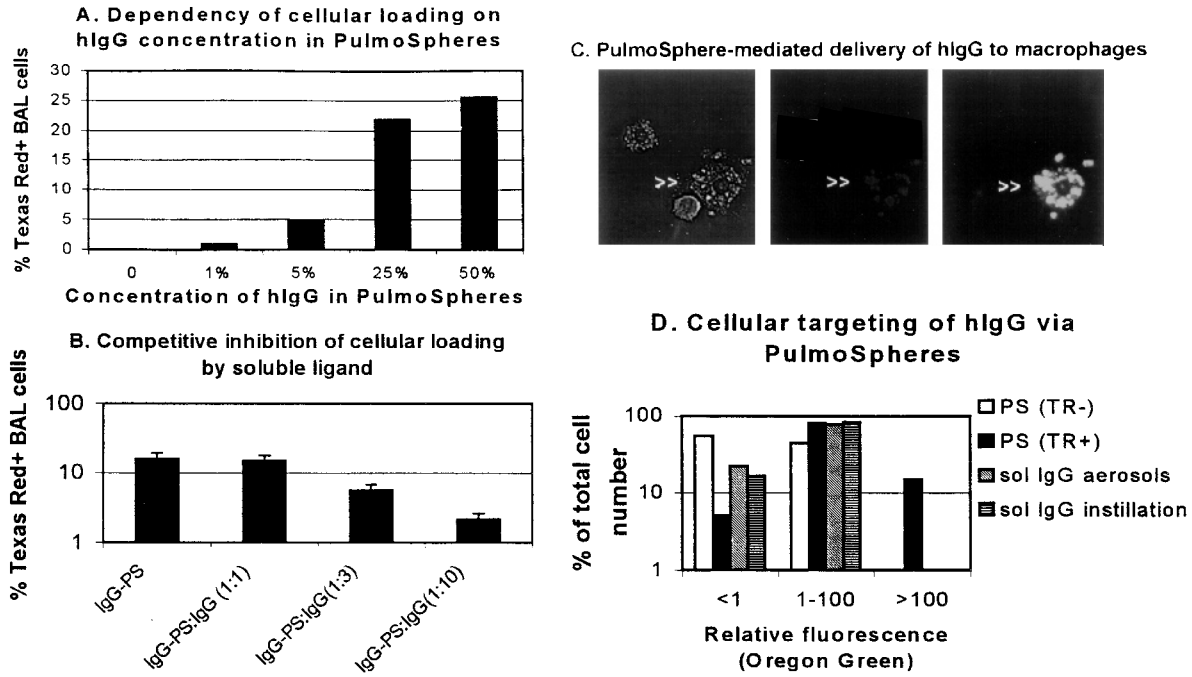

Fig. 6. Uptake of hIgG-loaded *PulmoSpheres* by bronchoalveolar macrophages. Dependency of uptake on the hIgG content of *PulmoSpheres* (A) and competitive inhibition of uptake by non-formulated, soluble hIgG (B). In the experiment described in panel A, bronchoalveolar macrophages were pulsed with *PulmoSpheres* tagged with Texas Red and loaded with various amounts of hIgG. The percentage of Texas Red$^+$ cells after 1 hour incubation is shown as function of % hIgG loaded into *PulmoSpheres*. In the experiment described in panel B, similar amounts of tagged *PulmoSpheres* (50% hIgG) were incubated for 1 hour with macrophages in the presence of increasing amounts of soluble hIgG. On the abscisa, 1:1, 1:3 and 1:10 represent the molar ratios of hIgG provided as *PulmoSpheres* versus non-formulated. The results are expressed as means ± SE of triplicates, of % Texas Red$^+$ cells. (C) Macrophage uptake of Texas Red-*PulmoSpheres* loaded with Oregon Green tagged hIgG. The same field comprising three cells (two of them exhibiting macrophage morphology) is shown by fluorescent microscopy in different conditions: left panel - normal light; intermediate and right panels, corresponding to filters for Texas Red and Oregon Green, respectively. Data is representative for two independent experiments with at least 200 cells/experiment inspected. (D) *In vivo* cellular uptake of hIgG delivered via *PulmoSpheres* or saline was measured by double-color flowcytometry of lavaged cells. The results are represented as % cells with given green fluorescent intensity. First two bars: cells lavaged from *PulmoSpheres*-treated animals and gated for red fluorescence (first bar: Texas Red$^-$ and second bar, Texas Red$^+$ cells). The next two bars correspond to cells harvested from animals treated with dose-matched non-formulated hIgG via liquid aerosols or instillation.

cellular uptake of hIgG-*PulmoSpheres* was inhibited by non-formulated, soluble hIgG in a dose-dependent manner (Fig. 6B). Pulsing alveolar macrophages with Texas Red-microspheres loaded with Oregon Green-tagged hIgG, showed co-localization of the tags in the same cells (Fig. 6C), consistent with the receptor-facilitated delivery of *PulmoSpheres*. We have administered Texas Red-*PulmoSpheres* loaded with Oregon Green-hIgG to rats by dry-powder inhalation via the tracheal route. Flowcytometric analysis of lavaged cells retrieved 1 hour after the treatment showed that more hIgG was internalized into Texas Red$^+$ cells (32% of lavaged cells) compared to Texas Red$^-$ cells (Fig. 6D), supporting the mechanism of *PulmoSphere*-mediated delivery of IgG. Furthermore, direct comparison with the profiles of cells retrieved from animals treated with matched doses of hIgG in saline, identified a subpopulation of Texas Red$^+$ phagocytic cells that displayed intense green fluorescence (15% of Texas Red$^+$ cells, with 100–1000 relative fluorescence) and was specific for the *PulmoSphere*-treated animals (Fig. 6D).

Together, these results show that formulated hIgG facilitates the uptake of antigen-loaded *PulmoSpheres* by antigen presenting cells, thus providing a potential explanation for the enhanced immunity (Figs. 3, 4, 5).

DISCUSSION

We have shown that the *PulmoSpheres* formulation technology provides a potential platform for delivery of immunoglobulins to or via the respiratory tract. The following findings support our conclusion: (a) the immunoglobulins retained their structural and functional integrity after formulation; (b) *in vitro* models and *in vivo* experiments showed release of immunoglobulins from *PulmoSpheres* and significant systemic bioavailability; (c) *in vivo* experiments demonstrated local and systemic immune responses mounted against foreign epitopes on formulated immunoglobulins.

The integrity of immunoglobulins after formulation in *PulmoSpheres* was confirmed by capture ELISA, showing that both light and heavy chain epitopes were present on the same molecule. These data combined with the SDS-PAGE and Western blot assays (Fig. 2) confirmed the integrity of the immunoglobulins after formulation in *PulmoSpheres*. *In vitro*

experiments showed that immunoglobulins are rapidly released from *PulmoSpheres* once added to an aqueous environment (Fig. 1). The results pinpointed that potential differences may exist with respect to the release kinetics, depending on the formulated compound. Characteristics like molecular weight and lipophilicity may play important roles in this regard. The observation that delivery via the trachea into the lungs was associated with a greater than 10-fold higher bioavailability as compared to intranasal administration (Fig. 2), supports the concept that immunoglobulin translocation is favored in the lower respiratory tract. That might be due to the increased surface area of delivery in the lower respiratory tract.

The lack of correlation between the systemic bioavailability and the immune responses raised to xenotypic epitopes of non-formulated and formulated hIgG (Figs. 3, 4, 5), suggests an immune modulatory effect of the formulation. Human IgG administered in saline via either the respiratory (data not shown) or intravenous routes, resulted in equal or higher systemic bioavailabilities as compared to hIgG in *PulmoSpheres*, yet they triggered lower immune responses. An intrinsic adjuvant effect of the excipients of *PulmoSpheres* was ruled out by the lack of enhanced immunity to empty microspheres co-administered with non-formulated hIgG (Fig. 5). Similar to other experimental systems using non-opsonized particles (18) we did not observe enhanced cytokine (TNF-α, IFN-γ, IL-10, IL-4, IL-6) production by splenocytes or alveolar macrophages exposed to empty *PulmoSpheres*. Thus, these observations implicate the antigen presenting cells and lymphocytes associated with the respiratory tract, in the immune response to hIgG loaded *PulmoSpheres*. The enhanced immunity (Figs. 3, 4, 5) may be explained by effective delivery to cells of the immune system via interaction between hIgG and Fc receptors (Fig. 6). The observed delayed kinetics of release of hIgG from *Pulmo Spheres* (Fig. 1) facilitates the targeting that is dependent on the interaction between IgG and FcR (Fig. 6). In addition, the aggregation of hIgG in the lipid shell may increase the avidity of interaction between *PulmoSpheres* and antigen presenting cells leading to their activation. Notably, previous studies characterized the ability of alveolar macrophages to internalize foreign antigens and to present them in secondary lymphoid organs to specific T cells (19). Based on more recent studies, complex regulatory roles were revealed for alveolar macrophages, consisting in selective inhibition of IL-2 dependent proliferation but not other functions of T cells (20). Finally, the presence and function of FcR for IgG (Fcγ-R) on alveolar macrophages was previously documented (18,21), consistent with our results regarding IgG mediated internalization of engineered *PulmoSpheres*. Our *in vivo* data that compared the efficiency of IgG loading by *PulmoSpheres* versus control saline, identified a subset of phagocytes effectively targeted by microspheres, that displayed approximately 10–100 higher uptake of hIgG (Fig. 6D). Thus, the enhanced immunity to formulated hIgG was not due to an increased systemic bioavailability (Fig. 2), but more likely to an effective targeting of antigen to APC (Fig. 6), resulting in increased T helper activity (Fig. 3) and enhanced IgG production (Figs. 3, 4).

The structure and size of *PulmoSpheres* may resemble that of low density polymer (poly[lactic acid-*co*-glycolic acid]) particles designed and reported previously by Edwards *et al.* (22). Similar to their observation, our studies showed that non-IgG *PulmoSpheres* were not readily phagocytosed by macrophages (Fig. 6). More recently, Ben-Jebria *et al.* (23) reported that sustained protection from bronchoconstriction was achieved with albuterol formulated in DPPC-based porous particles, suggesting decreased phagocytosis of particles closely related to *PulmoSpheres*. However, we have defined a specific strategy to greatly enhance the uptake of *PulmoSpheres* by antigen presenting cells, by incorporating a specific ligand - IgG (Fig. 6). This correlated with and may explain the enhanced immunity triggered by formulated IgG. In support of this conclusion, we showed that co-administration of soluble hIgG with 'empty' PulmoSpheres did not lead to enhanced immunity or uptake by antigen presenting cells (Fig. 5/Results).

There are two implications for the results we observed regarding the enhanced immunity triggered by xenotypic immunoglobulins encapsulated in microspheres when delivered to the respiratory tract. First, the results underscore the importance of minimizing the immunological mismatch when immunoglobulin therapy or prophylaxis is being considered. Secondly, this observation has implications for strategies aimed at inducing, increasing or modulating immune responses by using immunoglobulin constructs carrying foreign (8) or self (10) epitopes. The mere formulation of such constructs in hollow-porous microspheres devoid of intrinsic adjuvant activity, followed by non-invasive administration to the respiratory tract, may result in profound immunological effects.

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