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In vivo efficacy of dendrimer–methylprednisolone conjugate formulation for the treatment of lung inflammation

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ABSTRACT

Dendrimers are an emerging class of nanoscale intracellular drug delivery vehicles. Methylprednisolone (MP) is an important corticosteroid used in the treatment (through inhalation) of lung inflammation associated with asthma. The ability of MP–polyamidoamine (PAMAM) dendrimer conjugate to improve the airway delivery was evaluated in a pulmonary inflammatory murine model that was based on an 11-fold enhancement of eosinophil lung accumulation following five daily inhalation exposures of sensitized mice to the experimental allergen, ovalbumin. MP was successfully conjugated to PAMAM-G4-OH dendrimer yielding 12 MP molecules per dendrimer, and further solubilized in lysine carrier. Five daily trans-nasal treatments with the carrier alone, free MP, and MP–dendrimer at 5 mg kg^{-1} (on a drug basis) did not induce additional lung inflammation, although free MP decreased baseline phagocytic cell recoveries by airway lavage and tissue collagenase dispersion. MP treatments alone decreased ovalbumin-associated airway and tissue eosinophil recoveries by 71 and 47%, respectively. Equivalent daily MP dosing with MP–dendrimer conjugate further diminished these values, with decreases of 87% and 67%, respectively. These findings demonstrate that conjugation of MP with a dendrimer enhances the ability of MP to decrease allergen-induced inflammation, perhaps by improving drug residence time in the lung. This is supported by the fact that only 24% of a single dose of dendrimer delivered to the peripheral lung is lost over a 3-day period. Therefore, conjugation of drugs to a dendrimer may provide an improved method for retaining drugs within the lung when treating such inflammatory disorders as asthma.

1. Introduction

Asthma is a chronic inflammatory disorder of the airways that is associated with inflammation, airway hyperresponsiveness and airflow limitation that causes recurrent episodes of wheezing and airway obstruction of varying degrees. Antigen stimulation of airway mast cells and histamine release has been implicated in the early asthma reaction that is characterized by bronchoconstriction and airway edema likely to be responsive to bronchodilators such as β-adrenergic drugs like albuterol. On the other hand, the airway hyperresponsiveness associated with late asthma reaction and chronic asthma has been more closely linked to alterations in airway barrier function, remodeling and the mucosal presence of both neutrophil and eosinophil granulocytes (Chand et al., 1990; Holgate et al., 2006; Roberts et al., 1999). More recent investigations have also focused on the possible direct role of a small number of submucosal T-lymphocyte subsets in influencing airway smooth muscle function (Franchimont, 2004; Hahn et al., 2003). The main agents used for the treatment of asthma are β_2 agonists and corticosteroids. β_2 agonists provide bronchodilatory effects that help relieve airway obstruction associated with the early asthma reaction. Inhaled glucocorticoids such as methylprednisolone (MP) are effective anti-inflammatory agents and are the preferred treatment method for patients with persistent asthma at all levels of severity (Chand et al., 1990; Elenkov, 2004; Franchimont, 2004). Corticosteroids suppress airway inflammation, airway hyperresponsiveness, and are known to prevent asthmatic symptoms. Using higher doses of corticosteroids provides little additional benefit and increases the risk of side effects such as hypertension, osteoporosis, skin thinning, and adrenal suppression. Prolonged use of systemic corticosteroids can also lead to significant undesirable effects such as depression of the immune response leading to an increased susceptibility to infections, risk for peptic ulcers,

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myopathies, and more importantly, growth retardation in children (Shinwell et al., 2000). More recently, there are concerns that neonates that received post-natal systemic corticosteroids for the treatment of bronchopulmonary dysplasia have a higher incidence of cerebral palsy, impaired neurodevelopment and smaller brain mass as shown by magnetic resonance imaging (Wilson-Costello et al., 2009; Barrington et al., 2001; Halliday and Ehrenkranz, 2001; Eventov and Shinwell, 2008). Corticosteroids can be administered by inhalation, oral, and parenteral (subcutaneous, intramuscular and intravenous) routes. The major advantage of delivering drugs directly into the airways by inhalation is the potential for localized high concentrations that can be delivered very effectively to the airways. However, while it was previously thought that inhaled corticosteroids had minimal to no systemic side effects, studies have shown otherwise (Rao et al., 1999; Kannisto et al., 2004).

The use of inhaled corticosteroids would be greatly improved if doses can be reduced and systemic absorption decreased by targeted delivery. Existing methods of MP delivery are not tissue-specific which results in drug levels that are inconsistent, short-lived efficacy, and in many cases adverse effects. Therefore, a mechanism by which drugs are retained near their target site within the lung is needed to reduce the required dose and control systemic effects. We hypothesized that using a dendrimer–drug conjugate delivery system could address these issues while providing a sustained local drug concentrations. Dendrimers are a unique class of synthetic nanometer sized polymers. As robust three-dimensional structures they can be tailored to attach and carry drug molecules to specific tissues for efficient targeting. The surface of the dendrimers can be modified by attaching antibodies, ligands, or polyethylene glycol molecules to tailor the surface properties of the dendrimers in order to make them more specific to certain tissues and organs while regulating drug release. Dendrimers such as the one used for this study are non-immunogenic and exhibit low to no toxicity (Esfand and Tomalia, 2001). One potential approach that generated a lot of interest in macromolecular drug delivery involves the change in molecular weight and overall physical properties of the drug or pro-drug by conjugation which can significantly affect not only drug bio-distribution but also alter their cellular level of performance (Navath et al., 2008; Kurtoglu et al., 2009; Wang et al., 2009). Previous work from our group (Khandare et al., 2005; Kolhe et al., 2004, 2006) showed that dendrimer–methylprednisolone can be successfully conjugated and the synthetic conditions optimized.

In this study, we explored suitable methods of formulation and investigated both the potential of such a conjugate to diminish allergen-induced inflammation as well as identify possible adverse inflammatory reactions resulting from repeated treatments. The allergen exposure model system used in this investigation involved the assessment of the effects of daily trans-nasal treatments of the lungs of sensitized mice with increasing doses of methylprednisolone and dendrimer–methylprednisolone conjugate during a 5 day period of lung inflammation development brought about by daily inhalation exposures to the experimental allergen, ovalbumin. We found that there were no adverse inflammatory reactions associated with these treatments alone and that conjugation of the methylprednisolone with the dendrimers enhanced the anti-inflammatory effects of this glucocorticoid.

2. Materials and methods

2.1. Materials

Polyamidoamine (generation-4) dendrimer with amine end group (PAMAM-G4-NH_2) (MW = 14,200 Da) and hydroxyl end group (PAMAM-G4-OH) were purchased from Dendritech, Midland, MI. Methylprednisolone (MP) (M_w = 374.5 Da), fluorescent probe, fluorescein isothiocyanate (FITC) (M_w = 390), dicyclohexylcarbodiimide (DCC) (M_W = 206), 4-dimethylaminopyridine (DMAP) (M_W = 122.17 Da) and N-hydroxybenzotriazole (HOBt) (M_W = 135.1 Da) were purchased from Sigma–Aldrich. Glutaric acid (GA) (M_W = 132 Da) was purchased from Matheson Coleman and Bell Co. Dialysis membrane of molecular weight cut-off of 1000 Da was purchased from Spectra/Pore. Dimethyl sulfoxide was purchased from Fisher Scientific and all other chemicals used were analytical grade.

2.2. Preparation of conjugate

The dendrimer surface has a high density of functional groups, but the steric hindrance at the surface can limit achieving high drug payloads. For this reason, the dendrimer–drug conjugates were prepared using a 2-step procedure: (1) synthesis of glutaric acid (spacer) and methylprednisolone conjugate and (2) synthesis of PAMAM-G4-OH-glutaric acid–methylprednisolone conjugates (Fig. 1).

2.2.1. Synthesis of glutaric acid (GA) and methylprednisolone conjugate

Glutaric acid (42 mg, 0.534 mmol), DCC (110 mg, 0.534 mmol) and DMAP (catalytic quantity) were dissolved in anhydrous dimethyl sulfoxide (DMSO) in a 25 ml round bottom flask. The reaction mixture was stirred for 45 min to activate the carboxyl group of the glutaric acid. Methylprednisolone (200 mg, 0.534 mmol) was added to the reaction mixture and stirred for 72 h while the precipitation of dicyclohexylurea (DCU) was observed. The DCU was filtered to obtain a clear solution. The solvent was evaporated to obtain methylprednisolone-glutarate with some amount of unreacted glutaric acid. The product was washed with water to remove unattached glutaric acid.

2.2.2. Synthesis of PAMAM-G4-OH-glutaric acid (GA)–methylprednisolone (MP) conjugates

PAMAM-G4-OH and MP-GA conjugate ($M_w \sim 488.5$ Da) from the previous reaction were dissolved in anhydrous dimethyl sulfoxide (DMSO) separately. DCC (110 mg, 0.534 mmol) and HOBt (catalytic

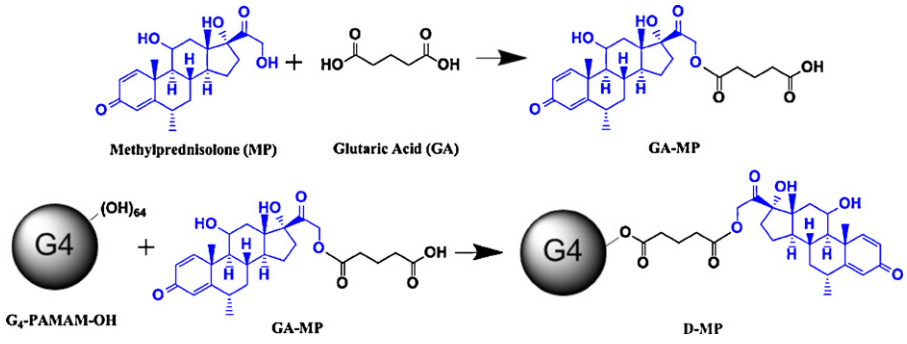

Fig. 1. Synthesis scheme of PAMAM-G4-OH-MP conjugate.

quantity) were added to MP-GA solution. The reaction mixture was stirred for 45 min to activate the unreacted carboxylic acid group of the MP–GA conjugate. PAMAM-G4-OH (0.007 mmol, 100 mg) was added to the reaction mixture and stirred for 72 h while the precipitate of DCU was observed. The reaction mixture was filtered to remove DCU. The filtrate was then dialyzed against DMSO for three days by replacing DMSO daily (dialysis membrane cut-off 1000 Da) to remove unreacted compounds. The dialyzed product was dried under vacuum to obtain the conjugate. ^{1}H NMR was used to characterize the conjugate with further verification with MALDI-TOF. The conjugation ratio was identified as being 12 molecules of MP per molecule of dendrimer.

2.2.3. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (MALDI-TOF)

The conjugate was dissolved in DMF at 1 mg/ml and 2,5-dihydroxybenzoic acid (DHB) matrix was dissolved in 50:50 (v/v) acetonitrile:water mixture at 20 mg/ml concentration. 2 μl of conjugate solution was mixed with 20 μl of DHB solution and 1 μl of the sample was spotted on a Bruker Daltonics MALDI plate. The sample was laser irradiated in a Bruker Daltonics MALDI instrument for molecular weight estimation.

2.3. Synthesis of FITC-labeled dendrimers

Generation 4 PAMAM dendrimer with amine surface functionality was dissolved in dimethyl sulfoxide (DMSO). Fluorescein isothiocyanate (FITC) was dissolved in DMF and added to the dendrimer solution slowly to prevent precipitation. The isothiocyanate group of FITC reacts with amine surface group of PAMAM dendrimer and the reactivity of these two groups are very high. The reactants were stirred for 24 h and then dialyzed using 1000 Da cut-off dialysis membrane to remove any free FITC. The dialysate solvent was evaporated under reduced pressure at 35 °C. ^{1}H NMR was used to characterize the conjugate and further verified with UV–vis absorbance spectroscopy.

2.4. Conjugate formulation using lysine complexation

200 mg of lysine was dissolved in methanol and a drop of hydrochloric acid solution was added. 45 mg of dendrimer–drug conjugate containing 10 mg of methylprednisolone was also dissolved in methanol and sodium hydroxide was added. Both the lysine and conjugate solutions were mixed and stirred for 1 h. The solvent was evaporated under reduced pressure and water was added to dissolve the conjugate. The sample was centrifuged at 10,000 rpm for 15 min to separate the undissolved conjugate. The complexation steps were repeated until almost all of the conjugate was dissolved.

2.5. Animal studies

Specific pathogen free female BALB/c mice weighing 20–25 g were obtained from Hilltop Laboratory Animals Inc. (Scottsdale, PA). Animals were maintained in high efficiency particulate filtered air with free access to food and water. Sensitization of all animals was achieved by intraperitoneal (IP) injections of 10 μg of ovalbumin (Grade V, Sigma Chemical Co., St. Louis) and 2 mg aluminum hydroxide suspended in 0.2 ml of sterile isotonic saline on day 1 and day 14 followed by exposures to either saline (control) or ovalbumin aerosols that were started 21 days from the first IP ovalbumin treatment. Animals were treated by a transnasal method while under isoflurane in oxygen anesthesia. Single aliquots of 15 μl were applied to the nares of anesthetized mice while being held in the vertical position. Samples were rapidly taken into the lungs and animals fully recovered from the anesthetic within 1–2 min. Administration of Evans Blue dye in sterile isotonic saline (0.5%, w/v) was used to evaluate the method, demonstrating relatively even distribution to the peripheral parenchymal regions of the lung. Subsequent Na_2SO_4 digestion of the parenchymal lung tissue followed by centrifugation and spectrophotometric analyses of the supernatant at 620 nm demonstrated a dye recovery of 42 ± 3% (±SEM, $n = 4$) of the administered dose. Although higher deposition is possible using other instillation methods, this relatively non-invasive procedure was chosen for use in this study due to suitability for the repeated drug administration protocol.

Mice were treated with either single 15 μl aliquots containing increasing concentrations of 6α-methylprednisolone 21-hemisuccinate sodium salt (0, 1.25, 2.5, and 5 mg kg^{-1} body wt) solubilized in a carrier containing 100 mg lysine and 200 mg of (2-hydroxypropyl)-β-cyclodextrin per ml sterile water (Sigma Chemical Co., St Louis, MO); or 15 μl equivalent doses of methylprednisolone (MP) bound to dendrimer (0.24 mg MP/mg of dendrimer) in carrier; or 15 μl of the carrier alone. Each administration was followed by a 15 μl isotonic saline wash. Mice were treated daily beginning 1 day before and 3 h following each of 5 daily exposures to either ovalbumin or saline aerosols.

Sensitized mice were exposed for 1 h per day for up to 5 days to either an ovalbumin aerosol generated from a 1% ovalbumin solution in sterile isotonic saline or saline alone (control) using a collision nebulizer and a nose-only exposure system that provides a relatively mono-dispersed aerosol with a mean aerodynamic diameter of 0.25 μm (CH Systems, New York). Air flow was adjusted to provide an aerosol concentration of 25 mg ovalbumin/m^3. Twenty-four hours following the last exposure, mice were sacrificed for the assessment of changes in lung inflammation.

The protocols used in this project were approved by the Wayne State University's Institutional Animal Care and Use Committee (IACUC), in accordance with all U.S. government guidelines. The facilities including those for animal exposure are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

2.5.1. Recovery of FITC-labeled dendrimers

The parenchymal lung tissue from mice treated with fluorescein isothiocyanate (FITC) labeled dendrimer in carrier complex was homogenized in 2 ml 3N NaOH and left in the dark for 72 h to dissolve tissue elements, prior to centrifugation to remove undissolved proteins. The resulting clear colored solutions were then analyzed at 492 nm and compared with standard curves generated by adding known amounts of the FITC-labeled dendrimers to untreated lung homogenates processed concurrently with lung homogenates from treated mice. With the additional use of internal standards, this method corrected for color quenching that was due to the presence of dissolved lung tissue elements.

2.5.2. Determination of inflammatory state

As previously described for rats and guinea pigs (DeLorme et al., 2002; Schultheis and Bassett, 1994), ventilated lungs of anesthetized mice were first perfused with warm isotonic saline (37 °C) via the pulmonary artery to remove blood components, prior to the collection of bronchoalveolar lavages (BAL) samples with 0.8 ml aliquots of phosphate-buffered saline (PBS) (pH. 7.4) containing 3 mM EDTA. The cells recovered from 5 serial lavages were combined and the resulting cell populations subjected to differential analyses following staining with modified Wright's stain for identification of macrophages, lymphocytes, neutrophils and eosinophils. Results were reported as total BAL-recovered cells.

The remaining parenchymal tissue was isolated, weighed, sliced and then incubated in Dulbecco's modified Eagles medium containing collagenase (75 U/ml; Worthington Biochemical Corporation,

Lakewood, NJ), fetal calf serum (10%, v/v; Gibco, Grand Island, NY), and 50 U/ml DNase (Sigma), as previously described (DeLorme et al., 2002; Schultheis and Bassett, 1994). A total cell count was conducted and differential cell analyses performed after separate staining with modified Wright's stain for neutrophils, eosinophils and lymphocytes and non-specific esterase staining for the identification of the combined monocyte and macrophage population (Li et al., 1973).

2.5.3. Statistical analyses

Data from these experiments were analyzed by one way or two-way analyses of variances with comparisons of means using either Dunnett's or Student–Newman–Keul's multiple range tests with a level of significance of $P < 0.05$. Logarithmic transformations of the data were conducted using Hartley's test when variances were found to be heterogeneous.

3. Results

3.1. Synthesis and characterization of PAMAM-G4-OH-MP conjugates

Glutaric acid (GA) was used as a spacer for the conjugation of methylprednisolone to dendrimer which increases the reactivity of the high molecular weight steroidal drug with the dendrimers (Khandare et al., 2005). The α-hydroxyl group of methylprednisolone was conjugated to the carboxyl group of glutaric acid. The other end of glutaric acid carboxyl group was conjugated with the hydroxyl of PAMAM-G4-OH. ^{1}H NMR and MALDI were used to estimate the drug payloads on the conjugate. NMR spectrum of the methylprednisolone–dendrimer conjugate is shown in Fig. 2. The peaks at δ 5.8, δ 6.2 and δ 7.3 ppm corresponded to three olefinic protons of methylprednisolone drug, and the peaks between δ 2 and δ 4 ppm corresponds to 985 protons of dendrimer (Wiwattanapatapee et al., 2003). Using the integration values of these peaks, the number of drug molecules attached onto the dendrimer was calculated. The ratio of the integration of the drug peaks (δ 5.8, δ 6.2 and δ 7.3 ppm) to that of the dendrimer peak (δ 2–δ 4 ppm) estimated that 12 molecules of MP were attached per molecule of dendrimer. To further verify this result, the dendrimer and the conjugate were estimated by MALDI-TOF. Free PAMAM-G4-OH molecular weight was determined as 13,800 Da and the MP–PAMAM-G4-OH had 19,473 Da molecular weight (Fig. 3), which corresponded to approximately 12 MP molecules per molecule of dendrimer. The drug payload calculated

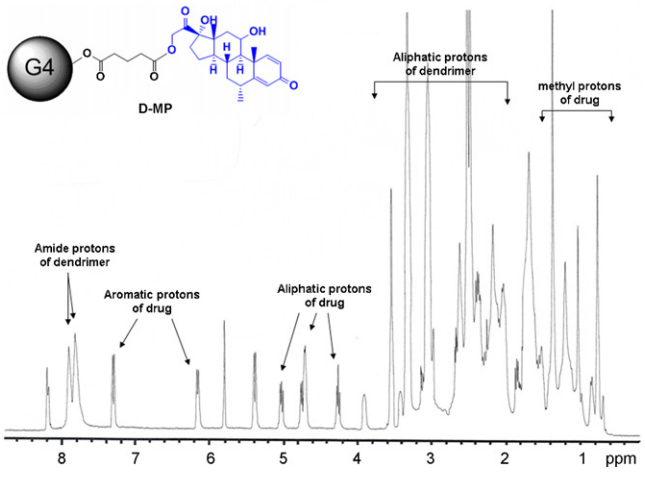

Fig. 2. ^{1}H NMR spectrum of PAMAM-G4-OH-MP conjugate.

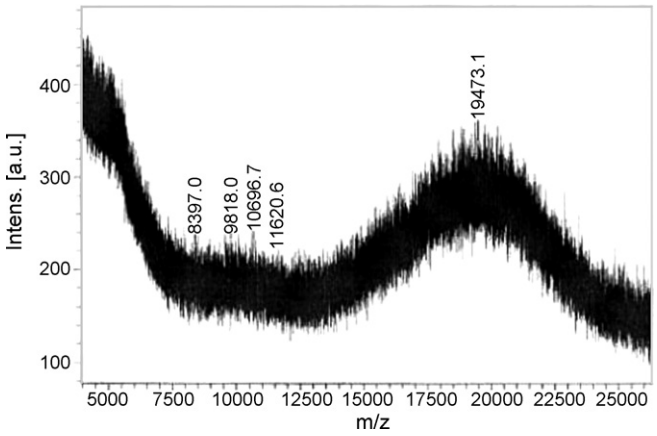

Fig. 3. MALDI spectrum of PAMAM-G4-OH-MP conjugate.

from both NMR and MALDI correlated well and for all future calculations a drug payload of 12 molecules per molecule of dendrimer was used. To check the purity of the conjugate, TLC of both the conjugate and free drug was performed in 10% methyl alcohol and 90% chloroform. The pure drug MP and MP-GA conjugate moved in the TLC plate but pure PAMAM-G4-OH and the conjugate of MP–PAMAM-G4-OH did not move in the TLC plate. The TLC results showed no free drug was present in the conjugate.

3.2. Formulation of the PAMAM-G4-OH-MP conjugate

While PAMAM dendrimers are water soluble, MP is a highly water insoluble drug. Previous work on this conjugate suggested that, if a large number of methylprednisolone molecules are conjugated to one molecule of dendrimer, the water solubility of the conjugate will be reduced. On the other hand, higher payloads of drug are crucial in achieving good efficacy and also to keep the minimum concentration of free amine functional groups of dendrimer in the formulation. For this purpose the conjugate used for the formulation had 12 molecules of methylprednisolone per molecule of dendrimer. However, the resulting conjugate had low solubility in water, and to improve the water solubility of these conjugates, two formulation strategies were examined: cyclodextrin encapsulation and lysine complexation. Although the cyclodextrin solubilized the conjugate, the complex resulted in significant lung inflammation when administered to mice at the concentration used in the formulation. However, lysine complexation did not induce any non-specific inflammatory effects on the lung. Almost 200 mg of lysine was used to dissolve 10 mg equivalents of MP–dendrimer conjugate in water. The formulation was stable as determined by high speed centrifugation. Intranasal administration of 15 μl at concentrations of up to 200 mg/ml lysine alone proved to have no additional inflammatory effect on the lung than that seen on treatment with isotonic saline as carrier (data not shown).

3.3. Evaluation of PAMAM dendrimer for intranasal drug delivery applications

FITC-labeled dendrimer in lysine carrier was used to examine the initial deposition and retention in the lung parenchyma after a single trans-nasal administration. Free FITC at a dose equivalent to the amount conjugated with amine terminated G4-PAMAM dendrimer was used as control. Lungs were then isolated at different times post-treatment to determine the initial drug-dendrimer conjugate delivery to the lung parenchymal tissue and the subsequent retention of the dendrimer over the following two weeks post-treatment. Fig. 4 demonstrates that a mean value of 43 ± 1%

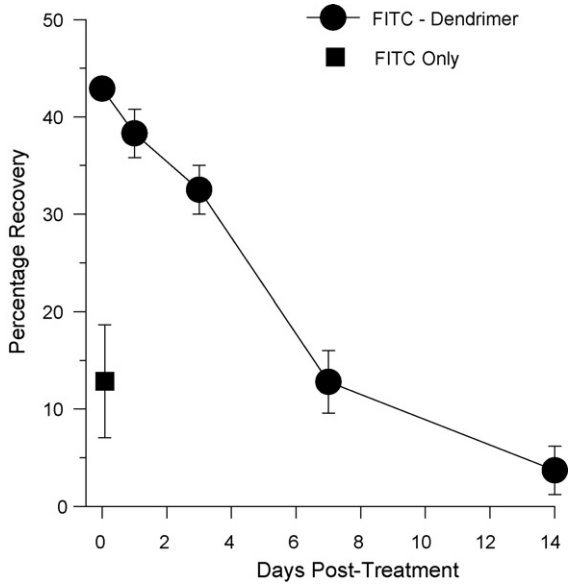

Fig. 4. Results represent mean ± SEM ($n = 3$) recoveries of FITC-labeled dendrimer–MP conjugate and FITC alone in lysine carrier at different times following single trans-nasal treatments.

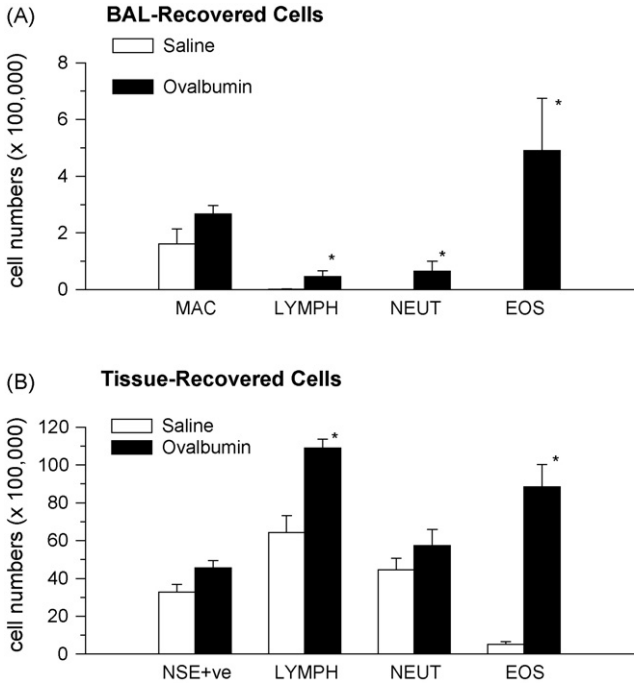

Fig. 5. Results represent the mean ± SEM ($n = 3$) recoveries of inflammatory cells recovered by bronchoalveolar lavage (A) and collagenase tissue dispersion (B) of lungs isolated from mice 24 h following the last of 5 daily 1 h exposures to either saline or ovalbumin aerosols. *Significantly different than results obtained with carrier alone with $P < 0.05$.

of the applied dose of dendrimer was recovered within the lung immediately following treatment. This value was comparable to the deposition of Evans Blue dye used to establish the trans-nasal method of lung administration, indicating deposition was unaffected by the presence of the dendrimer. Although this initial deposition value was not significantly reduced within the first 24 h, by 3 days post-treatment, the mean value of recovered FITC-labeled dendrimer was beginning to be diminished reaching 12 and 4% of the administered dose by 7 and 14 days post-treatment, respectively (Fig. 4). In contrast, FITC alone in the same carrier rapidly left the parenchymal region of the lung as demonstrated by only 30% of the administered FITC dose being recoverable from the lung parenchyma within 20–30 min of administration and a mean recovery value of 12% 2 h post-administration (Fig. 4). These results support the hypothesis that when small molecular weight compounds like FITC are conjugated with dendrimers, their residence time will increase significantly, encouraging the potential use of dendrimers to prolong the retention time of conjugated drugs within such tissues as the lung.

3.4. Allergen-induced lung inflammation

In the model of allergen-induced inflammation used in this study, mice were exposed daily to either saline or ovalbumin aerosol for 1 h. Twenty-four hours following the fifth daily exposure, lung inflammation was assessed by examining the cells recovered by extensive BAL and collagenase dispersion of the remaining parenchymal tissue (Fig. 5A and B). A total mean BAL-cell recovery from saline-exposed mice of $1.72 \pm 0.45 \times 10^5$ cells (±SEM, $n = 4$) was mainly identified as alveolar macrophages and although less than 0.03×10^5 lymphocytes were detected, neither neutrophils or eosinophils were identified. In contrast, ovalbumin-exposed mice demonstrated a 5.5-fold increase in total BAL-cell recoveries associated with significant enhancements in macrophages and lymphocytes, with major recoveries of both neutrophils and eosinophils (Fig. 5A). Recovery of tissue inflammatory cells was achieved by collagenase dispersion, demonstrating a total number $148 \pm 17 \times 10^5$ inflammatory cells recovered per lung (±SEM, $n = 4$), identified as being 22% non-specific esterase positive cells, representing the combine population of interstitial and marginated monocytes and macrophages, 43% lymphocytes, 30% neutrophils, and 4% eosinophils (Fig. 5B). Less than 1% of recovered cells were unidentifiable, and these most likely represented non-inflammatory cells surviving the collagenase digestion procedure. Although, ovalbumin-exposure significantly enhanced the tissue recoveries of lymphocytes and eosinophils ($P < 0.05$) consistent with the above BAL data, no statistical significant enhancements in the collagenase recoveries of non-specific esterase positive and neutrophils cells could be demonstrated (Fig. 5B).

3.5. Effects of drug administration

Preliminary investigations demonstrated no effect on the baseline recoveries of inflammatory cells by BAL and collagenase tissue dispersion of the lungs isolated from mice exposed to saline aerosols for up to five days. However, the potential to cause non-specific inflammatory effects by repeated trans-nasal administrations with the carrier alone, and pure MP, and MP conjugated with dendrimer in carrier were assessed in saline-exposed mice using a MP dose of 5 mg kg^{-1} body wt (Fig. 6A and B). Carrier treatment alone did not significantly alter the BAL recoveries of alveolar macrophages and lymphocytes, although the carrier administration did result in the appearance of a few neutrophils and eosinophils not previously observed in untreated mice (Figs. 5A and 6A). The tissue recovery of lymphocytes was also enhanced by the carrier treatments alone (Figs. 5B and 6B). Interestingly, repeated treatments of the carrier with 5 mg/kg^{-1} body wt MP significantly reduced the baseline tissue recoveries of non-specific esterase positive monocytes and macrophages, neutrophils and eosinophils (Fig. 6B). However, although no additional effect on tissue eosinophil recoveries was observed following treatment with the dendrimer–MP conjugate, neutrophil recoveries were no longer significantly decreased from values obtained on treating with the carrier alone (Fig. 6B).

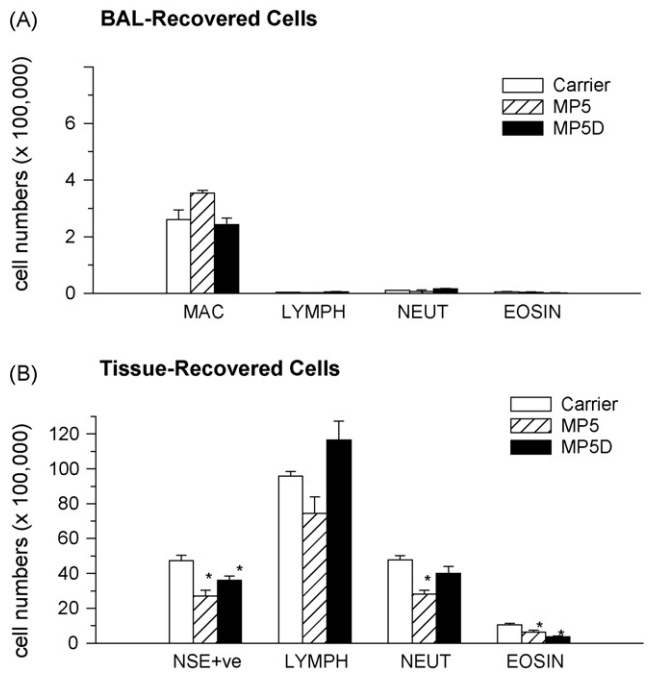

Fig. 6. Results represent the mean ± SEM ($n = 3$) recoveries of inflammatory cells recovered by bronchoalveolar lavage (A) and collagenase tissue dispersion (B) of lungs isolated from mice 24 h following the last of 5 daily 1 h exposures to saline aerosol and daily trans-nasal treatments with carrier alone, or carrier with either methylprednisolone (MP) or -MP–dendrimer conjugate (MPD) at a dose of 5 mg MP/kg^{-1} body wt/day. *Significantly different than results obtained with carrier alone with $P < 0.05$.

3.6. Ovalbumin-stimulated inflammation

As part of the same experimental series, the effects of daily MP and dendrimer–MP on ovalbumin-stimulated lung inflammation were assessed (Table 1 and Fig. 7). Although the BAL recoveries of lymphocytes associated with ovalbumin exposure were significantly diminished by MP treatments of 2.5 mg kg^{-1} body wt (data not shown), no further decreases were observed by treatments at the higher dose of 5 mg kg^{-1} body wt or with dendrimer–MP (Table 1). In contrast, increasing levels of MP treatment did not significantly decrease ovalbumin-associated neutrophil BAL accumulations, except when MP was conjugated to dendrimer where maximal decreases were observed at both the 2.5 mg (data not shown) and 5.0 mg kg^{-1} body wt MPD levels (Table 1). Treatment with MP alone or MPD did not have any effect on BAL recoveries of alveolar macrophages from ovalbumin-exposed mice (Table 1). Although treatment of MP alone significantly affected the ovalbumin-enhanced tissue lymphocyte recoveries, the recovery of neutrophils and the non-specific esterase positive combined monocyte and macrophage populations were unaffected by both MP and MP–dendrimer treatments (Table 1).

In contrast, the BAL and tissue recoveries of eosinophils, representing the major infiltrating inflammatory cell associated with the ovalbumin exposure, demonstrated significantly decreased recoveries with daily treatment with MP alone and MPD (Fig. 7). Significantly decreased BAL recoveries of eosinophils were achieved with treatments of 5.0 mg kg^{-1} body wt of MP alone. However a significant decrease of BAL recovery of eosinophils was observed at a lower concentration of 2.5 mg kg^{-1} body wt when MP conjugated with the dendrimer was used (Fig. 7A). Comparison of the results obtained using the 5 mg kg^{-1} body wt dose of MP alone also demonstrated that a further significant decrease in eosinophil recovery was possible when the MP was conjugated to the dendrimer (Fig. 7A). Tissue recoveries of eosinophils were also decreased by treatments with MP alone (Fig. 7B), that on comparisons at the higher MP dose of 5 mg kg^{-1} body wt, demonstrated a statistically significant further decrease when the MP was conjugated to dendrimer (MPD).

4. Discussion

In this study, we have demonstrated methods for generating and formulating a dendrimer conjugate with the anti-inflammatory corticosteroid drug MP. To our knowledge, this is the first study where a dendrimer drug conjugate was investigated using a lung exposure method based on trans-nasal administration. Following trans-nasal administration of the solution, this dendrimer was found to be retained within the lung with a half-life time of approximately 5 days (Fig. 4). Repeated daily administrations for 5 days showed that the MP–dendrimer (MPD) conjugate did not cause any observable non-specific inflammatory reactions within the lung as indicated by the lack of any enhanced recoveries of inflammatory cells by either bronchoalveolar lavage or by collagenase dispersion of parenchymal tissue (Fig. 6). Using a previous model of allergen-induced lung inflammation, increased anti-inflammatory MP efficacy was demonstrated when MP was conjugated with the dendrimer (Fig. 7).

The high payload of methylprednisolone covalently bound to dendrimer required the use of lysine complexation as a carrier system in order to achieve high solubility. Preliminary studies with relatively less soluble formulations demonstrated rapid clearance from the lung, possibly due to macrophage clearance of insoluble aggregates. In this study, the same levels of lysine were used in the carrier for the trans-nasal delivery of MP alone and of the conjugate that had a MP to dendrimer molecular ratio of 12 for comparison purposes. Repeated trans-nasal administration of saline-exposed mice demonstrated that these treatments did not result in any non-specific inflammatory effects (Fig. 6), suggesting that administration of dendrimer drug conjugate would not be expected to cause any adverse reactions. However, possible interference with specific host defense mechanisms of the lung might be considered for inclusion in future investigations. An observed decrease in baseline endogenous levels of marginated and interstitial inflammatory

Table 1
Inflammatory cell recoveries from drug-treated ovalbumin-exposed mice.

	BAL-recovered cells			Tissue-recovered cells		
	Macrophages ($\times 10^5$)	Lymphocytes ($\times 10^5$)	Neutrophils ($\times 10^5$)	NSE positive ($\times 10^5$)	Lymphocytes ($\times 10^5$)	Neutrophils ($\times 10^5$)
Carrier only ($n = 12$)	3.31 ± 0.29	0.91 ± 0.21	0.83 ± 0.20	63.88 ± 6.42	94.45 ± 7.64	49.28 ± 3.81
MP (5 mg kg^{-1} body wt) ($n = 6$)	3.70 ± 0.32	0.19 ± 0.01*	0.58 ± 0.26	52.66 ± 6.20	58.33 ± 7.09*	58.46 ± 10.57
MP–dendrimer (5 mg kg^{-1} body wt) ($n = 3$)	3.84 ± 0.83	0.18 ± 0.03*	0.22 ± 0.04*	33.43 ± 7.81	68.62 ± 2.27	37.70 ± 1.76

Results represent mean ± SE of the number of cells recovered per lung by bronchoalveolar lavage (BAL) and subsequent collagenase tissue dispersion of lungs recovered from ovalbumin- exposed mice treated with either carrier alone, methylprednisolone (MP) or methylprednisolone–dendrimer (MPD). Since no differences were observed between the cyclodextrin- and lysine-based carriers, the results have been combined for presentation purposes.

* Significantly different than corresponding results obtained from carrier-treated animals with $P < 0.05$.

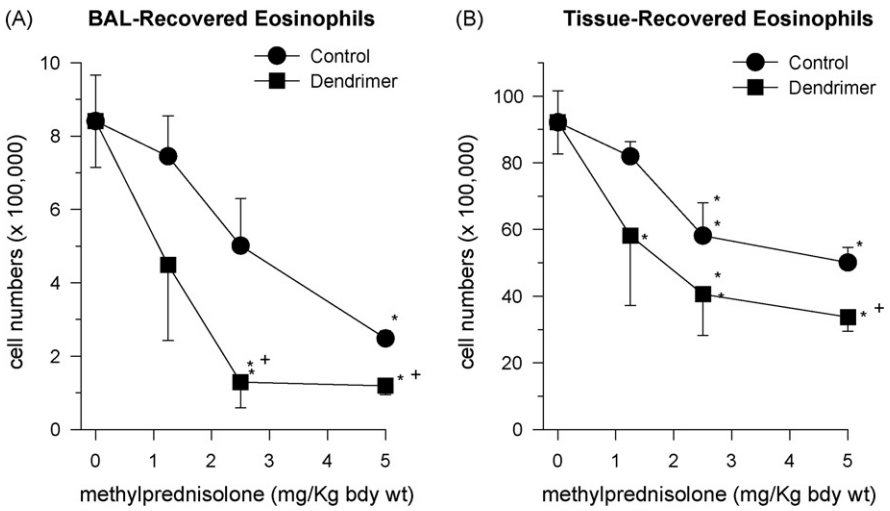

Fig. 7. Results represent the mean ± SEM recoveries of inflammatory cells recovered by bronchoalveolar lavage (A) and collagenase tissue dispersion (B) of lungs isolated from mice 24 h following the last of 5 daily 1 h exposures to ovalbumin aerosol and daily trans-nasal treatments with carrier alone ($n = 12$), or carrier with either methylprednisolone (MP) ($n = 6$) or MP–dendrimer conjugate (MPD) ($n = 3$) at a doses of 0, 1.25, 2.5 and 5 mg MP/kg^{-1} body wt/day. *Significantly different than results obtained with carrier alone and +significantly different than results obtained from corresponding MP treatments, with $P < 0.05$.

cells on treatment with both MP and MP–dendrimer conjugate treatments were consistent with the anti-inflammatory effect of methylprednisolone (Fig. 6).

In contrast to previous models of allergen-induced inflammation that have examined the early and late phase of acute asthmatic reactions in response to single allergen exposures (Chand et al., 1990; Evans et al., 1997), the mouse model used in this study employed daily repeated exposures to relatively low levels of allergen aerosol. Such a model permits the study of how five daily drug treatments might affect the development of allergen-associated inflammatory lesions over an extended period of time. In this system, within the first two daily allergen exposures tissue lymphocyte numbers maximally increase with a concomitant enhancement in marginated and tissue eosinophils. However, these latter cells do not begin to appear in the BAL fluid until after 3 daily exposures, after which time both airway and tissue eosinophil recoveries continue to increase on a daily basis with a concomitant enhancement in non-specific airway constriction on aerosol exposure to the bronchoconstrictor methacholine (data not shown). Although increased lymphocytes and neutrophils are observed after 5 daily exposures (Fig. 5), the largest enhancement in airway and tissue-recovered cells was observed to be eosinophils. This is consistent with a Th2 lymphocyte driven inflammatory response to allergen, observed in the late inflammatory phase of an acute asthmatic reaction (Chand et al., 1990; Roberts et al., 1999).

In the present study, daily trans-nasal treatment with a preparation of MP up to 5 mg kg^{-1} body wt, diminished this inflammatory cell accumulation, consistent with similar studies obtained with oral- dosing of allergen-exposed mice with the corticosteroid dexamethasone (Fig. 7) (Birrell et al., 2003). However it should be noted that recent studies from our laboratory using the same allergen exposure model have demonstrated that daily trans-nasal dosing with MP at levels of greater than 5 mg kg^{-1} body wt reversed the anti-inflammatory reaction. Such results support the hypothesis that under certain conditions, the anti-inflammatory effects of glucocorticoids might be reversed by their potential ability to stimulate components of the adaptive immune system (Elenkov, 2004; Franchimont, 2004).

Our observations that maximal inhibition of allergen-induced eosinophil infiltration into the lung airways can be achieved at a lower MP dose when bound to a dendrimer supports the hypothesis that the corticosteroid was retained within the lung tissue for an extended period of time. Since a single dose of dendrimer demonstrated a half-life time within the lung parenchyma of approximately 5 days (Fig. 4), these observations collectively support the use of dendrimer drug conjugates to reduce the amount of drug used for controlling chronic inflammatory lung disorders such as asthma, as well as perhaps decreasing the frequency of drug administrations. However, it should be noted that the use of the trans-nasal daily dosing procedure in this study only accounted for 40–50% of the dose being delivered to the distal lung, raising the possibility that the material may have been deposited in the upper airway where it is subsequently cleared or swallowed and absorbed via the gastrointestinal tract. More effective methods for drug delivery method, might well demonstrate further enhancements in the efficacy of the MP–dendrimer conjugate.

In summary, we have shown that methylprednisolone can be successfully conjugated to PAMAM-G4 dendrimers and the intranasal administration of methylprednisolone–dendrimer conjugate was effective in reducing the ovalbumin-induced airway inflammatory response in a mouse model. More importantly, a lower concentration of drug was needed to reduce the inflammatory reaction when MP was conjugated with dendrimer. In this study, the administration of MP–dendrimer conjugate did not produce any undesired effects such as non-specific inflammation. Furthermore, the FITC–dendrimer conjugate had a significantly longer residence time in the lungs. This has the potential to support the use of dendrimer–drug conjugates to prolong transit time in tissues. In future studies, we plan to use fluorescent and radiolabeling to examine the lung distribution and retention of the MP–dendrimer conjugate following intranasal administration and to determine if systemic MP absorption also occurs with this formulation.

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