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Effect of betamethasone phosphate loaded polymeric nanoparticles on a murine asthma model

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ABSTRACT

Although inhaled steroids are the treatment of first choice to control asthma, administration of systemic steroids is required for treatment of asthmatic exacerbation and intractable asthma. To improve efficacy and reduce side effects, we examine the effects of betamethasone disodium phosphate (BP) encapsulated in biocompatible, biodegradable blended nanoparticles (stealth nanosteroids) on a murine model of asthma. These stealth nanosteroids were found to accumulate at the site of airway inflammation and exhibit anti-inflammatory activity. Significant decreases in BALF eosinophil number were maintained for 7 days with a single injection of nanosteroids containing 40 µg BP. Airway responsiveness was also attenuated by the injection of stealth nanosteroids. A single injection of 40 µg of free BP and 8 µg of free BP once daily for 5 days did not show any significant effects. We conclude that stealth nanosteroids achieve prolonged and higher benefits at the site of airway inflammation compared to free steroids.

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1. Introduction

Asthma is an inflammatory disease that is frequently controlled using inhaled steroids [1,2]. Inhaled corticosteroids represent the treatment of choice to control asthma and avoid serious systemic side effects. However, systemic administration of glucocorticoids is still required for intractable asthma or asthmatic exacerbation [3].

Glucocorticoids are one of the most effective drugs for the treatment of airway inflammation, but systemic application should be used only when necessary and in as low a dose as possible because of the high incidence of serious adverse effects such as an immunological suppression and diabetes [4–7]. When glucocorticoids are administered intravenously, they are distributed throughout the whole body and rapidly cleared [5]. For the treatment of asthmatic attack, high and frequent administration is required to achieve an

effective concentration at the inflamed target sites in the lungs [7,8].

Novel drug delivery systems are under investigation to enhance drug localization to the target site and achieve sustained drug release. Liposome-encapsulated steroids have been developed and used for asthma and arthritis, with confirmed efficacies [9,10]. However, allergic reactions to liposome preparations have also been reported [11]. To avoid some of these problems, the technique of encapsulation of drugs with nanoparticles has recently become available [12].

Ishihara et al. first developed betamethasone disodium 21-phosphate (BP) encapsulated in nanoparticles of poly(D,L-lactic acid (PLA)) homopolymers, which can resolve inflammation [13–15]. But nanoparticles of poly PLA homopolymers also accumulate in the liver, since they are cleared by the liver Kupffer cells. To improve the distribution to the targeted site, blended nanoparticles of poly PLA homopolymers and polyethylene glycol (PEG)-block-PLA copolymers encapsulating BP were developed as stealth nanosteroids that possess uncharged, hydrophilic and non-immunogenic properties [16,17]. PEG modification reduces opsonization and prevents interaction with the mononuclear phagocyte system [16–19]. In addition, blending of PLA homopolymers and PEG-block-PLA copolymers improves drug loading and controls stealth property [16,17]. In the present study, we examine the

Abbreviations: BP, betamethasone disodium 21-phosphate; PLA, poly D,L-lactic acid; PEG, polyethylene glycol; DEA, diethanolamine; EE, encapsulation efficiency; Cy, Cyanidin; TR-FIA, time-resolved fluoroimmunoassay; HE, hematoxylin and eosin staining; OD, optical density; Raw, airway resistance; Cdyn, dynamic compliance.

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anti-inflammatory activity and duration of effectiveness of stealth nanosteroids using a murine model of asthma.

2. Experimental procedures

2.1. Preparation and characterization of stealth nanosteroids

Nanoparticles were prepared using the oil-in-water solvent diffusion method, as reported previously [13,14,16]. In brief, a mixture (50 mg) of PLA homopolymers and PEG-PLA block copolymers was dissolved in 1 ml of acetone. A 500- μ l aliquot of 15 mg/ml diethanolamine (DEA) in acetone, 68 μ l of 1 M zinc chloride (pH 1.9), and 28 μ l of 350 mg/ml BP were added, then the mixture was allowed to stand for 30 min at room temperature. A 26-G needle was used to add the mixture dropwise to 25 ml of distilled water stirred at 1000 rpm at a rate of 48 ml/h. During diffusion of the organic solvent into water, nanoparticles formed rapidly along with the solidification of polymers. A 1-ml aliquot of 0.5 M citrate (pH 7.0) and 125 μ l of 200 mg/ml Tween 80 were immediately added to chelate BP–zinc complexes and enhance the stable dispersion of nanoparticles, respectively. Finally, nanoparticles were purified and concentrated by ultrafiltration (Centriprep YM-50; Amicon, Millipore, Bedford, MA, USA). Vehicle-only PLA and PEG-PLA nanoparticles without BP were also prepared as a control.

Particle size was determined using the dynamic light scattering method (FPAR-1000; Otsuka Electronics, Osaka, Japan). The drug loading of BP in nanoparticles was determined by HPLC (Agilent 1100 series, Agilent, Waldborn, Germany) with a ZORBAX Eclipse XDB-C18 column. The stealth nanoparticles used in this study comprised a 10% PEG blend (diameter, 116 ± 10 nm). Drug loading was 10.7 ± 1.0 wt.%. Cyanidin(Cy)7-dodecylamine conjugate was also prepared as described before [17].

2.2. Animals

BALB/c mice (8-week-old females weighing 20–25 g) obtained from SLC (Shizuoka, Japan) were used in this study. Animals were housed in a specific pathogen-free environment and allowed free access to food and water. All experimental protocols were approved by the Musashino University Animal Care Committee in accordance with the Guide for the Care and Use of Laboratory Animals.

2.3. Sensitization

Mice were initially immunized intraperitoneally twice with 10 μ g ovalbumin (OVA) and 2 mg aluminum hydroxide gel (on days 0 and 14), as previously reported with slight modifications [20]. After immunization, animals were subjected to inhalation of OVA (20 mg/ml) at a flow of 3 ml/min for 10 min every day, using an ultrasonic nebulizer (Omron, Tokyo, Japan). Mean particle diameter was 3.0 μ m. Saline-treated mice were used as controls. Each group consisted of 4–6 mice.

2.4. Drug treatment

Nanosteroids were administered intravenously through the tail vein immediately after the third inhalation of OVA, which is designated as day 0. OVA inhalation was subsequently continued. Free BPs were administered intraperitoneally and saline was administered intravenously. A previous study showed that 3 μ g of nanosteroids was effective in improving arthritis in a murine model [17]; however, our preliminary experiments in a similar model revealed that this dose was not sufficient. Previous reports showed that 1–3 mg/kg betamethasone is required to treat asthma in a

murine model [21,22]. For this reason, we used nanosteroids at a dose of 40 μ g per mice (2 mg/kg) in the present study. Free betamethasone was used single dose or continuous use once a day for 5 days.

2.5. Biodistribution of nanoparticles

After injection of nanosteroids, mice were sacrificed and lung, spleen and liver were excised. The organs were washed quickly with cold water and frozen immediately in liquid nitrogen. The tissues were extracted to assay BP concentration using a time-resolved fluoroimmunoassay (TR-FIA) kit for betamethasone (Shionogi & Co., Osaka, Japan), as described previously [13]. The detection limit of BP for this assay was 0.01 μ g/ml.

The accumulation was also evaluated by fluorescence imaging using a whole-body animal *in vivo* imaging system (Optix[®], GE Healthcare). Nanoparticles encapsulating Cy7-dodecylamine conjugate or free Cy7-dodecylamine were injected into the tail vein. After 24 h, mice were anaesthetized with sodium pentobarbital (45 mg/kg) intraperitoneally and placed on the chamber of the imaging system equipped with 750 nm filter.

2.6. Bronchoalveolar lavage fluid (BALF) cell analysis and histological examination

At 24 h after the last inhalation, each mouse was exsanguinated. BALF was obtained by incubating and washing the lung with 1 ml of saline until the recovered volume was 5 ml. For histological analysis, lungs were fully inflated at a pressure of 25 cm H₂O and fixed with 10% formaldehyde for hematoxylin and eosin (HE) staining. To quantify inflammation, an average of the number of infiltrated cells in 0.01 mm² of peribronchial region was counted using HE stained specimen according to the previously reported methods with some modification [9,23].

Concentrations of IL-13 and IL-4 in BALF were determined using an specific ELISA Quantizing kit (R&D Systems, Minneapolis, MN, USA and RayBiotech, Inc., respectively).

2.7. Assessment of airway responsiveness

Assessment of airway responsiveness was undertaken 24 h after the last OVA inhalation as previously reported with some modifications [20]. Briefly, anesthetized mice were tracheotomized and injected with pancuronium bromide. Animals were connected to a Harvard ventilator with 0.3 ml tidal volume and a respiratory rate of 150 breaths/min. Next, mice were placed in a whole-body plethysmograph (Buxco Electronic, Inc., Troy, NY) to measure airway resistance (Raw) and dynamic compliance (Cdyn) before and after inhalation of methacholine. Mice inhaled 25 mg/ml of methacholine for 3 min each using an ultrasonic nebulizer (Omron Co., Ltd, Tokyo, Japan) at a flow rate of 1.5 L/min.

2.8. Measurements of serum OVA-specific IgE

To measure OVA-specific IgE, we coated the plate with OVA, following incubation with samples diluted 10-fold. After washing, horse radish-labeled rat anti-murine IgE specific antibody (Morinaga, Kanagawa, Japan) was added to the plate as the second antibody. Color was developed and measured at 450 optical densities (OD).

2.9. Statistics

Kruskal–Wallis test and Dunn multiple comparison were used. Values of $p < 0.05$ were considered statistically significant. Data are expressed as means $\pm$ standard error of the mean (SEM).

3. Results

3.1. Time course of betamethasone concentration in the lung

After the third inhalation of OVA, nanoparticles with 40 μ g BP were administered intravenously through the tail vein. The concentration of BP in the lung was serially examined. BP was below detection level after 24 h of treatment with free BP (Fig. 1). In contrast, BP was detected in the lungs from mice injected with stealth nanosteroids even after 7 days ($p < 0.01$, compared to control and free BP). Nanosteroids released BP continuously in the lung. When nanoparticles with 40 μ g BP were administered to control naive mice, BP concentration was 0.17 ± 0.02 mg/kg at day 7 ($n = 6$, significantly less than that of OVA-treated mice, $p < 0.05$). We also examined the biodistribution of stealth nanoparticles using nanoparticles encapsulating Cy7-dodecylamine conjugate.

As shown in Fig. 2, nanoparticles significantly accumulated in the lung compared with free Cy7.

3.2. Anti-inflammatory effects of nanosteroids in the lung

To examine the anti-inflammatory effect of nanosteroids, we carried out BALF cell analysis and histological examination. Significant neutrophil accumulation was observed on day 1 of the OVA-treated asthmatic group. Significant eosinophil accumulation was observed from day 5 after inhalation of OVA. A single injection of 40 μ g of stealth nanosteroids significantly and consistently suppressed inflammatory cell accumulation in BALF from day 1 to day 7 (Fig. 3A, nanosteroids). In contrast, free BP (single injection of 40 μ g) did not exhibit any suppressive effects (Fig. 3A, BP). The suppressive effects of the stealth nanosteroids (single injection of 40 μ g) were comparable to free BP at 20 μ g/day for 5 days (Fig. 3B, frequent BP). Free BP (single injection of 40 μ g, Fig. 3A and B, BP) and 8 μ g/day for 5 days did not exhibit significant effects (Fig. 3B). The nanoparticles without steroids did not show any anti-inflammatory effects (data not shown).

Histological examination on day 7 showed cell infiltration around the airways and the venules (Fig. 4 OVA), which were attenuated in mice treated with stealth nanosteroids (single injection of 40 μ g, Fig. 4 nanosteroids). No significant differences between nanosteroids-treated and non-treated mice were observed

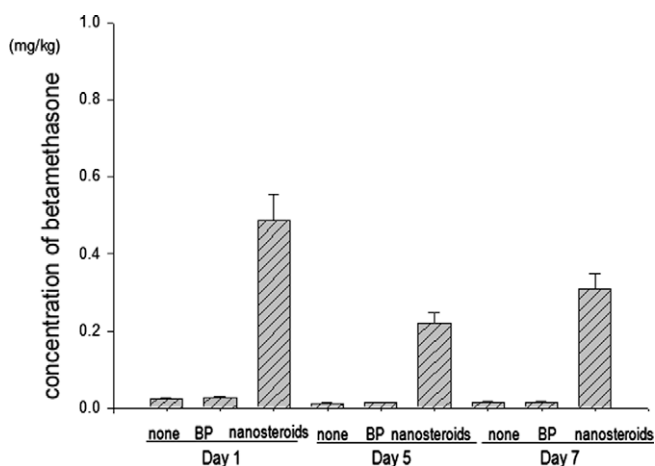


Fig. 1. BP concentration in the lung. OVA-treated mice were intravenously administered with nanosteroids (40 μ g BP) through the tail vein. Free BP was administered intraperitoneally and the saline was administered intravenously. Mean $\pm$ SEM (line) of six mice in each group is shown from Days 1, 5 and 7. The amount of BP in nanosteroids-treated mice was significantly higher than that of control and free BP-treated mice from day 1 to day 7 ($p < 0.01$).

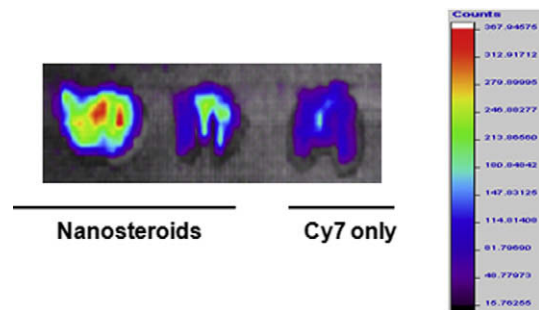


Fig. 2. Biodistribution of BP. Nanoparticles encapsulating BP and Cy7-dodecylamine conjugate or free Cy7-dodecylamine were injected into the tail vein of OVA-treated mice. The fluorescence images of the lungs after 24 h are shown using an imaging system at 750 nm.

in serum IgE (14.2 ± 2.3 versus 14.5 ± 1.2 U/ml), because nanosteroids were administered after systemic OVA immunization. These data suggest that stealth nanosteroids efficiently suppressed airway inflammation.

3.3. Inhibitory effects on IL-13 and IL-4 production

Next, we examined the effects on cytokine production. Interleukin (IL)-13 and IL-4 are known as a key cytokine for airway inflam-

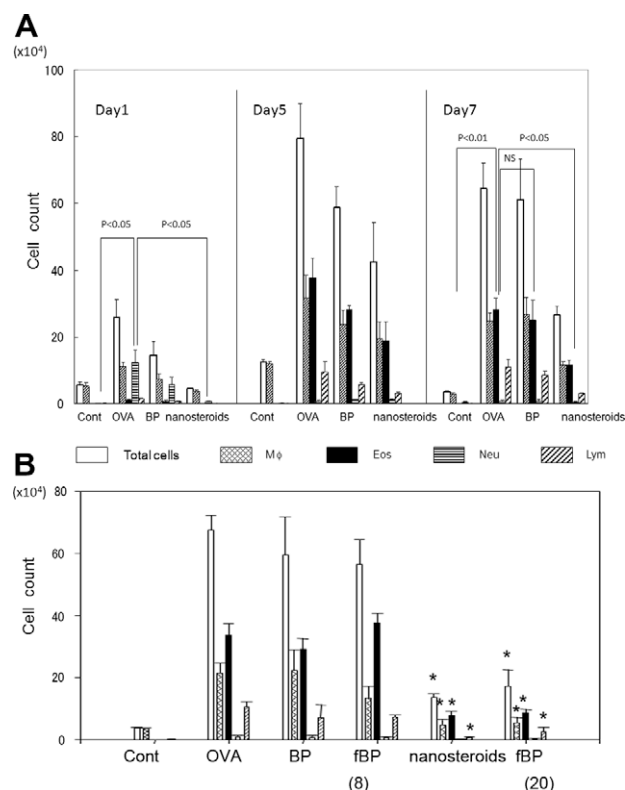


Fig. 3. BALF cell differential analysis. Mice were treated as described in the methods and the legend of Fig. 1. Cont indicates control naive mice, which were not immunized with OVA but treated with saline. All mice except controls were immunized with OVA and inhaled OVA. BALF cells were placed on glass slides using Cytospin. Slides were then stained with Diff Quik, and cell differentiation was assessed microscopically. The ordinate shows the number of cells in total BALF (5 mL). Each bar indicates mean $\pm$ SEM of five different mice. (A) Analysis of kinetics. BP indicates free BP (single injection of 40 μ g), nanosteroids indicate nanoparticles with 40 μ g BP. (B) Analysis of dose effects BP indicates free BP (single injection of 40 μ g), nanosteroids indicate nanoparticles with 40 μ g BP, fBP indicates frequent free BP injection, (20); 20 μ g/day for 5 days and (8); 8 μ g/day for 5 days. * indicated $p < 0.05$ compared to OVA.

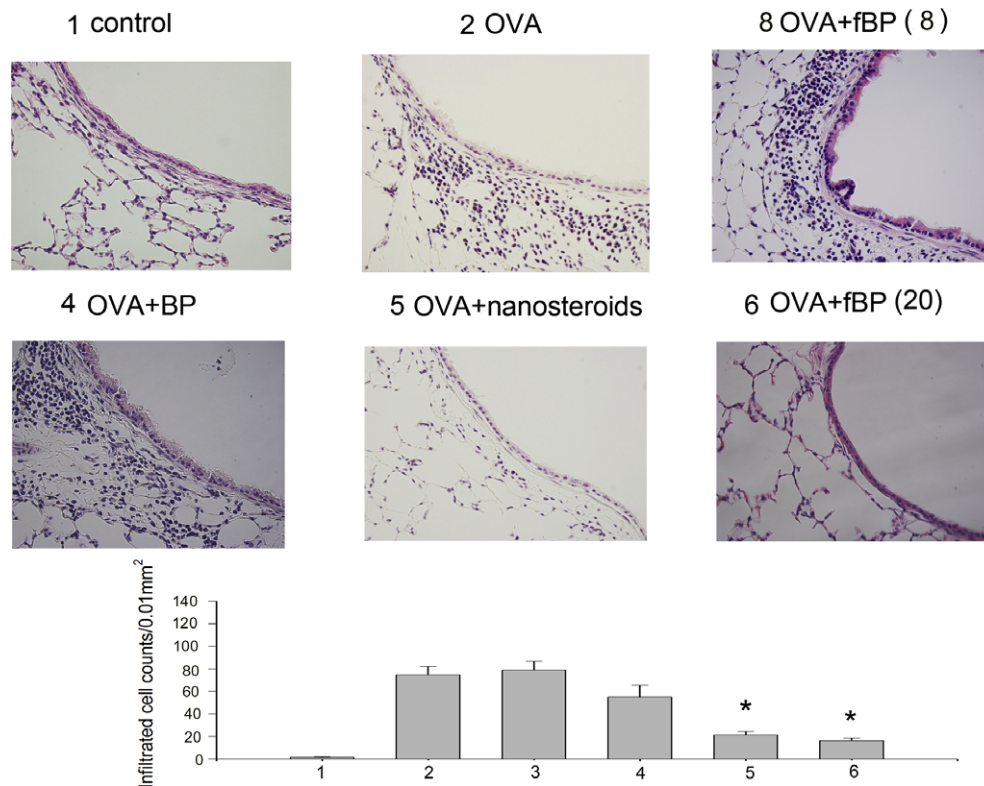


Fig. 4. Histological analyses by HE staining. Mice were sacrificed by exsanguinations. Lungs were intubated and slowly inflated at a pressure of 25 cm H₂O to avoid further damage to the lungs. Original magnification 400×. Each group indicates; 1, control; 2, OVA inhalation; 3, OVA + fBP (8):OVA inhalation + BP 8 µg/day for 5 days; 4, OVA + BP:OVA inhalation with free BP (single injection of 40 µg); 5, OVA + nanosteroids:OVA inhalation + nanosteroids (single injection of 40 µg); 6, OVA + fBP (20):OVA inhalation + BP 20 µg/day for 5 days. Lower panel shows an average of the number of infiltrated cells in 0.01 mm² of peribronchial region. Each bar indicates mean ± SEM of 10 different region.* indicated $p < 0.05$ compared to 2 OVA. X axis indicates the group described in upper panel.

mation in asthma. A single injection of stealth nanosteroids efficiently inhibited IL-13 and IL-4 production on day 7 (Fig. 5). The effects were comparable to free BP at 20 µg/day for 5 days (Fig. 5). However, the same dose of free BP did not show any effects.

3.4. Effects on airway hyperresponsiveness

Airway hyper responsiveness to methacholine is a characteristic of asthma. Cdyn (Fig. 6A) and airway resistance (Fig. 6B) were significantly suppressed by the administration of stealth nanosteroids

on day 7. Nanosteroids exhibited dose-dependent suppressive effects on airway hyperresponsiveness (Fig. 6B).

4. Discussion

We examined the therapeutic potential of polymeric nanoparticles encapsulating BP in a murine model of asthma. Our experiments revealed that stealth nanosteroids can achieve sustained anti-inflammatory activity. We showed that the number of infiltrated cells, as well as the level of IL-13 and IL-4 in BALF cells, significantly decreased up to 7 days after treatment with stealth

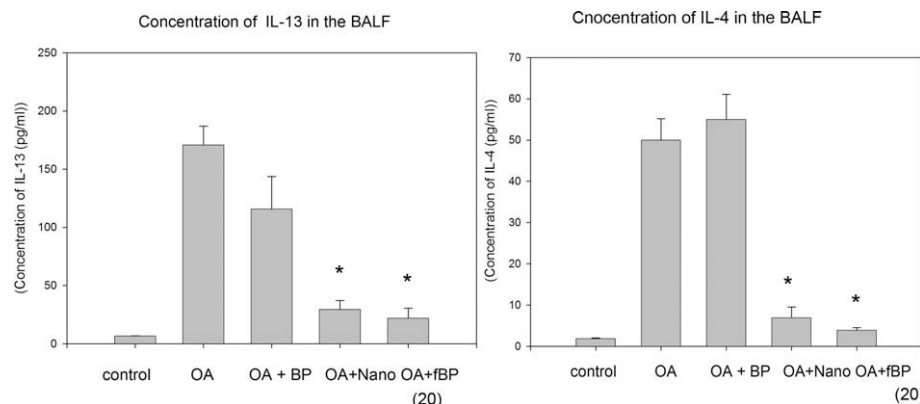


Fig. 5. IL-13 and IL-4 production. IL-13 and IL-4 content in BALF on day 7 was determined using ELISA. Each bar indicates mean ± SEM of 6 different mice. * indicated $p < 0.01$ compared to OVA. BP indicates free BP (single injection of 40 µg). nanosteroids indicate nanoparticles with 40 µg BP. fBP (20) indicates frequent free BP injection at 20 µg/day for 5 days.

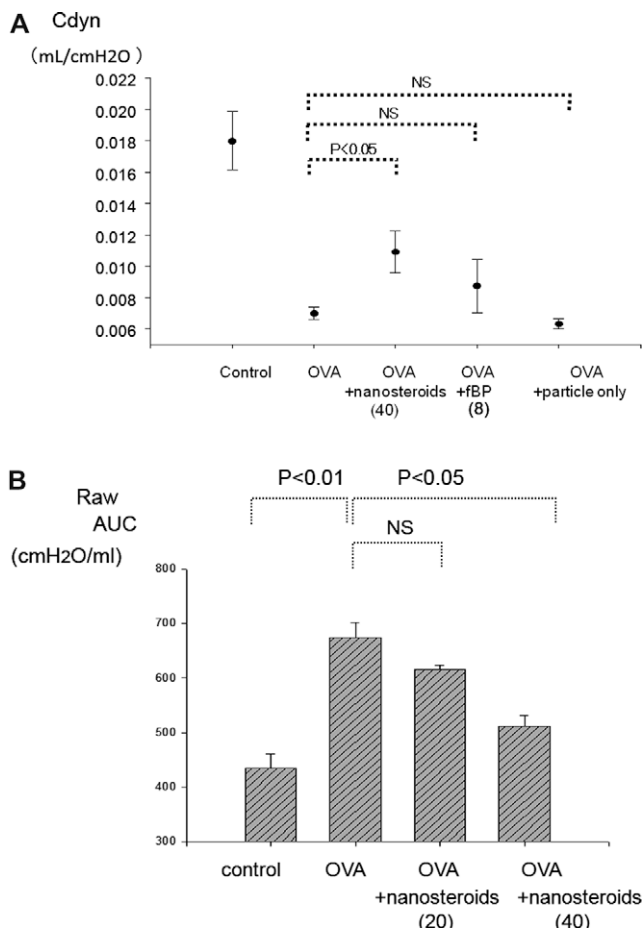


Fig. 6. Airway responsiveness. (A) Assessment using Cdyn. Mice were intubated and Cdyn and airway resistance was assessed before and after 25 mg/ml of methacholine, as described in the methods. Data are expressed as means $\pm$ SEM (line) of seven mice. The horizontal axis indicates: cont; control naive mice, OVA only; OVA + nanosteroids (single injection of 40 μ g), OVA + fBP; OVA + free BP 8 μ g/day for 5 days, OVA + nanoparticle only; OVA + nanoparticle without BP. (B) Assessment using Raw. Airway resistance from the beginning of 25 mg/ml of methacholine was measured for 3 min. Area under the curve (AUC) was calculated and expressed at the longitudinal axis. Data are expressed as means $\pm$ SEM (line) of four mice. Nanosteroids (20) indicates single injection of stealth nanosteroids with 20 μ g BP. Nanosteroids (40) indicate single injection of stealth nanosteroids with 40 μ g BP.

nanosteroids ($p < 0.05$ and < 0.01 , respectively). Stealth nanosteroids achieved an anti-inflammatory effect within 1 day and this effect was sustained for 7 days. A single intravenous injection of stealth nanosteroids was able to induce a strong, rapid, long-lasting therapeutic benefit in our asthma model. A single dose of nanosteroids at 40 μ g per mouse had a significant anti-inflammatory effect on asthma that was comparable to the frequent injection of free betamethasone (20 μ g per mouse (1 mg/kg)/day for five days).

Although mild asthma can be effectively-treated using inhaled steroids [24], severe asthma requires treatment with oral steroids also [3,25]. Disadvantages of the use of oral steroids include systemic side effects such as diabetes mellitus and osteoporosis [5,8]. To reduce these adverse effects, a reduction in dose while augmenting the positive effects of the steroids would be useful. Nanoparticles have the ability to enter the inflamed venule and accumulate within the inflamed tissue [26,27]. It is expected that nanoparticles would not be distributed throughout the body and would thus act more efficiently, resulting in dose reduction. Here, we achieved effective dose reduction by using nanosteroids. Inha-

lation of nanosteroids also provides the potential for further dose reduction in asthma therapy, and in support of this treatment option, liposome preparations of steroids are already in use [28–31].

To determine whether nanosteroids are effective to reduce side effects, we examined the levels of cholesterol, triglycerides, and glucose. We could not clarify the difference in this study, because side effects were not detected even after frequent injection of betamethasone (20 μ g/mouse for five days) (control vs. frequent injection of betamethasone: glucose, 123 ± 15 vs. 156 ± 16 mg/dl, NS; cholesterol, 88 ± 14 vs. 61 ± 5 mg/dl, NS). Since longer administration times of betamethasone are required to clarify its side effects [8,9,32], we plan to examine the potential side effects of this drug over an administration period of 8 weeks. The nanoparticles themselves did not cause any side effects over the administration time period in the present study. However, to clarify the potential side effects, these engineered particles should also be observed over a longer administration period [26].

Previous data have shown that non-stealth nanosteroids are effective, but accumulate in the liver and spleen resulting in their rapid removal from the circulation [14]. To improve drug delivery, stealth nanosteroids have been developed with biodegradable and long-circulating properties [16,17]. A single injection of the stealth nanosteroids results in remission of joint inflammation for 9 days [17]. Nanosteroids reduced macrophages and neutrophils accumulated at these inflamed joints [17]. Our study showed that stealth nanosteroids were also effective in the treatment of allergic inflammation, in which eosinophils are the main inflammatory cell type. Because nanosteroids were effective in the treatment of neutrophil inflammation as in the present study (Fig. 3A on day1) and in the previous study [17], stealth nanosteroids might also be effective in the treatment of severe asthma in which neutrophil infiltration is prominent and usually steroid-resistant [3,33]. To clarify this issue, further experiments using a neutrophil-dominant model are required. Another potential therapeutic target is airway remodeling. These improved steroids might be effective to treat airway remodeling and this notion is currently under investigation. In summary, we demonstrate that stealth nanosteroids have potential as a useful tool in improving the quality of life of steroid-dependent patients with severe asthma.

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