

Efficacy of liposomal budesonide in experimental asthma

Kameswari S. Konduri, MD,^a Sandhya Nandedkar, PhD,^a Nejat Düzgünes, PhD,^b
Vincent Suzara, BS,^b James Artwohl, DVM,^c Ralph Bunte, DVM,^c and
Pattisapu R. J. Gangadharam, PhD^c Milwaukee, Wis, San Francisco, Calif, and Chicago, Ill

Background: Inhaled corticosteroids, such as budesonide, attenuate the inflammatory response in asthma. However, patient noncompliance and side effects of available inhaled corticosteroids limit their use. Liposomes are currently used in medicine to deliver a variety of drugs.

Objective: The objective of our study was to determine whether weekly therapy with budesonide encapsulated in sterically stabilized (stealth) liposomes would be comparable to daily budesonide therapy in reducing allergic inflammation.

Methods: Ovalbumin-sensitized C57/Black 6 mice received aerosolized (1) budesonide encapsulated in stealth or conventional liposomes, administered weekly, (2) budesonide (without liposomes), administered either daily or weekly, or (3) empty stealth liposomes, administered weekly. All treatment groups were compared with sensitized untreated or unsensitized mice. Histopathologic examination of the lung tissues and measurements of eosinophil peroxidase activity, peripheral blood eosinophil counts, and total serum IgE levels were done weekly for 4 weeks.

Results: Weekly therapy with budesonide encapsulated in stealth liposomes was as effective as daily budesonide therapy in decreasing lung inflammation and lowering eosinophil peroxidase activity, peripheral blood eosinophils, and total serum IgE levels. In none of the other groups was there a significant decrease in the inflammatory parameters evaluated.

Conclusion: We conclude that weekly therapy with budesonide encapsulated in stealth liposomes is as effective as daily budesonide in reducing markers of lung inflammation in experimental asthma. This novel strategy offers an effective alternative to standard daily budesonide therapy in asthma and has the potential to reduce toxicity and improve compliance. (*J Allergy Clin Immunol* 2003;111:321-7.)

Key words: Budesonide, asthma, liposomes, lung inflammation, inhaled glucocorticoids, lung inflammation

Abbreviations used

BAL:	Bronchoalveolar lavage fluid
Daily-Bud:	Daily budesonide treatment
EPO:	Eosinophil peroxidase activity
OVA:	Ovalbumin
PB:	Peripheral blood
Sens:	Sensitized untreated
Wk-Bud:	Weekly treatment with budesonide only
Wk-C-Bud:	Weekly treatment with budesonide in conventional liposomes
Wk-Empty-S:	Weekly treatment with empty stealth liposomes
Wk-S-Bud:	Weekly treatment with budesonide in stealth liposomes

Asthma is the most common chronic illness in childhood, its cost being estimated at \$12 billion per year.¹ Current asthma therapy is directed at reducing pulmonary inflammation through use of anti-inflammatory drugs, such as inhaled corticosteroids.² However, the need for daily administration of inhaled steroids might lead to patient noncompliance and treatment failure. In addition, inhaled steroids have a short half-life in vivo and potential toxicity if higher doses are used.³⁻⁷

Liposomes are phospholipid bilayer vesicles that can encapsulate a variety of drugs; the liposome provides a repository for the drug to be released slowly so as to allow for a prolonged therapeutic effect. Budesonide is an inhaled steroid that is frequently prescribed for asthma and is also available in a nebulized form. We hypothesized that budesonide encapsulated in liposomes would decrease airway inflammation in asthma at lower doses and at less frequent dosing intervals in comparison with daily budesonide therapy.

Liposome-encapsulated antibiotics show increased efficacy in a variety of infectious diseases.⁸⁻¹¹ Liposomes have also been used for the delivery of aerosolized asthma medications, such as cromolyn sodium and albuterol sulfate.^{12,13} However, the potential role of liposome encapsulation in enhancing the efficacy of inhaled steroid preparations used in asthma remains unknown.

Liposomes are characterized by their lipid composition, surface charge, steric interactions, and number of lamellae. Conventional liposomes are composed of naturally occurring phospholipids, such as phosphatidylglycerol and phosphatidylcholine mixed with or without cholesterol. Although conventional liposomes can

From ^athe Department of Pediatrics, Medical College of Wisconsin, Milwaukee; ^bthe Department of Microbiology, University of the Pacific, San Francisco; and ^cthe Department of Internal Medicine and Biologic Resource Laboratory, University of Illinois, Chicago.

Funded by the Children's Research Center of Michigan at Children's Hospital of Michigan and the Children's Research Foundation of Wisconsin.

Presented in part at the AAAAI 57th Annual Meeting, March 16-21, 2001, New Orleans, La.

Received for publication September 23, 2002; revised October 27, 2002; accepted for publication October 31, 2002.

Reprint requests: Kameswari S. Konduri, MD, Medical College of Wisconsin, Division of Allergy and Immunology, 9000 West Wisconsin Avenue, PO Box 1997, Milwaukee, WI 53201.

© 2003 Mosby, Inc. All rights reserved.

0091-6749/2003 \$30.00 + 0

doi:10.1067/mai.2003.104

Asthma, rhinitis,
other respiratory
diseases

encapsulate a variety of drugs, they are recognized *in vivo* by the cells of the reticuloendothelial system and are cleared rapidly from the circulation.¹⁴ In addition, incorporation of triamcinolone or beclomethasone into conventional liposomes results in their rapid redistribution and leakage from liposomes into the medium.¹⁵⁻²⁰

In contrast to conventional liposomes, sterically stabilized "stealth" liposomes exhibit increased stability in plasma and decreased uptake by the reticuloendothelial system.²¹⁻²⁴ Although several studies have reported the use of conventional liposomes in asthma therapy,^{12,13,15-20} stealth liposomes have to our knowledge not been investigated as a carrier for the delivery of anti-inflammatory agents. In addition, there are no reports on the use of budesonide encapsulated in either stealth or conventional liposomes.

The objective of this study was to test the efficacy of weekly aerosol administration of budesonide encapsulated in stealth liposomes in a mouse model that reproduces the cellular and immunologic markers of inflammation in asthma.^{25,26} The efficacy of this novel drug delivery system was compared with that of standard daily budesonide therapy, weekly administration of budesonide alone, weekly administration of budesonide in conventional liposomes, and weekly administration of empty stealth liposomes.

METHODS

Animals

Six-week-old male C57Black 6 mice were purchased from Charles River Laboratories (Wilmington, Mass). The animals were provided with an ovalbumin-free diet and water *ad libitum* and were housed in an environment-controlled, pathogen-free animal facility. All animal protocols were approved by the Animal Care Committee of the University of Illinois at Chicago and the Medical College of Wisconsin and were in agreement with the National Institute of Health's guidelines for the care and use of laboratory animals.

Ovalbumin sensitization

The animals were sensitized with ovalbumin (OVA) through use of a modified protocol, as described by de Siquiera et al.²⁷ On day 0, each mouse was anesthetized with methoxyflurane given by inhalation. A fragmented heat-coagulated OVA implant was inserted subcutaneously on the dorsal aspect of the cervical region.

For a 10-day period (days 14-24), each mouse was given a 30-minute aerosolization of a 6% OVA solution on alternate days. This method of sensitization led to significant elevations in eosinophil peroxidase activity (EPO), peripheral blood (PB) eosinophils, and serum IgE levels, along with lung inflammation as seen on histopathologic examination, by day 24.²⁸

Treatment groups

Therapy was initiated on day 25, the day after the OVA sensitization was completed. Sensitized animals received nebulized treatments for 4 weeks, as follows: (1) budesonide (20 µg) encapsulated in stealth liposomes, administered once a week (the Wk-S-Bud group); (2) budesonide (20 µg) without liposome encapsulation, administered daily (standard therapy; the Daily-Bud group); (3) budesonide (20 µg) encapsulated in conventional liposomes, administered once a week (the Wk-C-Bud group); (4) buffer-loaded (empty) stealth liposomes, administered once a week (the Wk-Empty-S group); (5) budesonide (20 µg) without liposome encapsulation, administered once a week (the Wk-Bud group). Each of

the nebulization doses was given at a volume of 1 mL for 2 minutes through use of a chamber in which the mouse was allowed to breathe freely. All treatment groups were compared with either sensitized untreated or unsensitized (normal) mice.

The amounts of lipids used for the Wk-Empty-S group were based on the amount of lipid nebulized for each of the budesonide-encapsulated liposomes (1.39 µmol for the stealth liposomes and 3.19 µmol for the conventional liposomes). The dose of budesonide chosen was based on preliminary dose-response studies with 5 to 50 µg of budesonide.

Each day, 5, 10, 15, 20, or 50 µg of budesonide was administered via nebulization to groups of sensitized mice, and the dose-dependent effects on the inflammatory parameters were evaluated. These data were compared with data for either a group of sensitized untreated mice (the Sens group) or a group of unsensitized mice (the Normal group). A 20-µg dose of budesonide was shown on histopathologic examination to effectively decrease EPO activity in BAL, PB eosinophils, and inflammation of the lung tissues, along with other inflammatory parameters, without evidence of toxicity to the spleen, liver, bone marrow, or gastrointestinal tract. In addition, there were no granulomas or abnormalities in any of the tissues evaluated.

Each study group consisted of 20 mice and was followed for a 4-week period. Five animals from each treatment group and from each of the 2 control groups (Sens and Normal) were killed by means of an overdose of methoxyflurane inhaled 24 hours after the first treatments were given and then at weekly intervals for 4 weeks. At each time point, measurements of EPO in BAL, PB eosinophils, and total serum IgE levels were obtained and histopathologic examination of the lung tissues was performed.

Drugs and reagents

Budesonide for daily therapy was diluted from premixed vials (0.25 mg/mL) commercially available from Astra Pharmaceuticals (Wayne, Pa) and was administered via a Salter Aire Plus Compressor (Salter Labs, Irvine, Calif). Budesonide for encapsulation and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was purchased from Sigma Chemical (St Louis, Mo). Phosphatidylcholine, phosphatidylglycerol, and poly(ethylene glycol)-distearoylphosphatidylethanolamine were obtained from Avanti Polar Lipids (Alabaster, Ala). Cholesterol was purchased from Calbiochem (La Jolla, Calif). NaCl and KCl were purchased from Fisher Scientific (Pittsburgh, Pa).

Liposome preparation

Budesonide was encapsulated into either stealth (phosphatidylglycerol-phosphatidylcholine-poly(ethylene glycol)-distearoylphosphatidylethanolamine-cholesterol) or conventional (phosphatidylglycerol-phosphatidylcholine-cholesterol) liposomes through use of a protocol derived from that described by Gangadharam et al.⁹ A portion of the cholesterol used in control liposomes was replaced by budesonide (dissolved in chloroform-methanol [2:1]) during the preparation of the lipid mixture. Lipids were dried onto the sides of a round-bottomed glass flask or glass tube by rotary evaporation. The dried film was then hydrated by adding sterile 140 mmol/L NaCl and 10 mmol/L HEPES (pH 7.4) and vortexing. The resulting multilamellar liposome preparations were extruded 21 times through polycarbonate membranes (either 0.2 or 0.8 µm in pore diameter; Nuclepore, Pleasanton, Calif) through use of an Avestin extrusion apparatus (Toronto, Canada).

Histopathology observations

Histopathologic examination was performed on lungs that were removed and fixed with 10% phosphate-buffered formalin. Tissue samples were taken from the trachea, bronchi, large and small bron-

chioles, interstitium, alveoli, and pulmonary blood vessels. The tissues were embedded in paraffin, sectioned at a thickness of 5 μ m, stained with hematoxylin and eosin, and analyzed through use of light microscopy at a magnification of $\times 100$. Coded slides were examined by a veterinary pathologist, in a blinded fashion, for evidence of inflammatory changes, including (1) bronchiolar epithelial hyperplasia and wall thickening, (2) bronchiolar, peribronchiolar, and perivascular edema, and (3) accumulation of eosinophils, neutrophils, and mononuclear inflammatory cells. Each of the parameters evaluated was given an individual number score. The cumulative score was obtained through use of the individual scores; inflammation was designated as none (score, 0), mild (score, 1-2), moderate inflammation (score, 3-4), or severe inflammation (score, 5-6).

Eosinophil peroxidase activity in bronchoalveolar lavage fluid and peripheral blood eosinophils

When each mouse was killed, the trachea was exposed and cannulated with a ball-tipped 24-gauge needle. The lungs were lavaged 3 times with 1 mL PBS. All of the washings were pooled and the samples were frozen at -70°C . The samples were later thawed and assayed for determining EPO activity.

EPO in the BAL was assessed through use of a modification of the method described by Strath et al.²⁹ A substrate solution consisting of 0.1 mol/L sodium citrate, 0-phenylenediamine, and H_2O_2 (3%), pH 4.5, was mixed with BAL supernatants at a ratio of 1:1. The reaction mixture was incubated at 37°C , and the reaction was stopped by the addition of 4 N H_2SO_4 . Horseradish peroxidase was used as a standard. EPO activity (in international units per milliliter) was measured by spectrophotometric analysis at 490 nm. The percentages of eosinophils were obtained by counting the number of eosinophils in 100 white blood cells under a high-power field ($\times 100$) from the PB smears stained with Wright-Giemsa stain.

Total serum IgE

Ninety-six-well flat-bottom plates (Fisher Scientific) were coated with 100 μ L per well of 2 μ g/mL rat antimouse IgE monoclonal antibody (BD PharMingen, San Diego, Calif) and incubated overnight at 4°C . Serum was added at a dilution of 1:50 and incubated overnight at 4°C . Purified mouse IgE (k isotype, small b allotype anti-TNP; BD PharMingen) was used as the standard for total IgE. The samples were incubated for 1 hour with biotin-conjugated rat antimouse IgE (detection antibody purchased from Southern Biotechnology, Birmingham, Ala).

Data analysis

Data analysis was performed through use of the Student *t* test. *P* values of less than .05 were considered significant. Statistical analysis was performed through use of weekly serial measurements from each group. Cumulative data for the 4-week period for each study group are presented as means $\pm$ SEMs.

RESULTS

Over the 4-week period, there were no significant increases or decreases in inflammation within each group according to weekly measurements for all of the inflammatory parameters being evaluated.

Histopathology

Examples of lung tissues from the treatment groups are shown in Fig 1. Significant reduction in total lung histopathology score (Fig 2) was noted with weekly

treatments of budesonide encapsulated in stealth liposomes ($P = .020$) in comparison with what was seen in the sensitized untreated mice, and this reduction was similar to that seen with daily budesonide therapy ($P = .030$). Similar decreases were not observed with the weekly budesonide encapsulated in conventional liposomes treatment, the weekly budesonide treatment, or the weekly empty stealth liposomes treatment.

There was also a significant decrease in lung inflammation in the Wk-S-Bud group in comparison with the Wk-Empty-S group ($P = .0009$) and the Wk-Bud group ($P = .05$). There was no significant difference between the Daily-Bud group and the Wk-S-Bud group.

The lung tissues from the sensitized untreated mice had persistent and significant inflammation, including accumulation of inflammatory cells with considerable numbers of eosinophils in bronchiolar, peribronchiolar, and perivascular tissues, along with significant submucosal thickening and epithelial hyperplasia, during the 4-week period.

Eosinophil peroxidase activity

Weekly treatments with budesonide encapsulated in stealth liposomes significantly decreased the EPO activity ($P = .031$) in the BAL in comparison with what was seen in the sensitized untreated mice, and they were comparable to daily budesonide therapy ($P = .038$; Fig 3). The Wk-Bud ($P = .419$), Wk-Empty-S ($P = .213$), and Wk-C-Bud ($P = .366$) groups did not show any significant decreases in EPO activity.

Peripheral blood eosinophils

Therapy with weekly budesonide encapsulated in stealth liposomes ($P = .007$) and therapy with daily budesonide ($P = .001$) significantly decreased PB eosinophils in comparison with what was seen in the Sens group (Fig 4). None of the other treatment groups, including the Wk-C-Bud group, showed significantly decreased PB eosinophils in comparison with the Sens group.

Total serum IgE

Treatment with weekly budesonide encapsulated in stealth liposomes and treatment with daily budesonide significantly lowered the total serum IgE level ($P = .016$ and $P = .005$, respectively; Fig 5). The total serum IgE level was not significantly reduced in the Wk-C-Bud group or any of the other treatment groups in comparison with the Sens group.

DISCUSSION

In the present study, we demonstrated that budesonide encapsulated in stealth liposomes, given once a week, reduced inflammation as effectively as budesonide given once a day. Weekly treatments with free budesonide, budesonide encapsulated in conventional liposomes, and empty stealth liposomes did not have comparable effects.

Inhaled corticosteroids are the most commonly prescribed anti-inflammatory drugs in asthma therapy. However, the need for daily dosing might lead to problems of

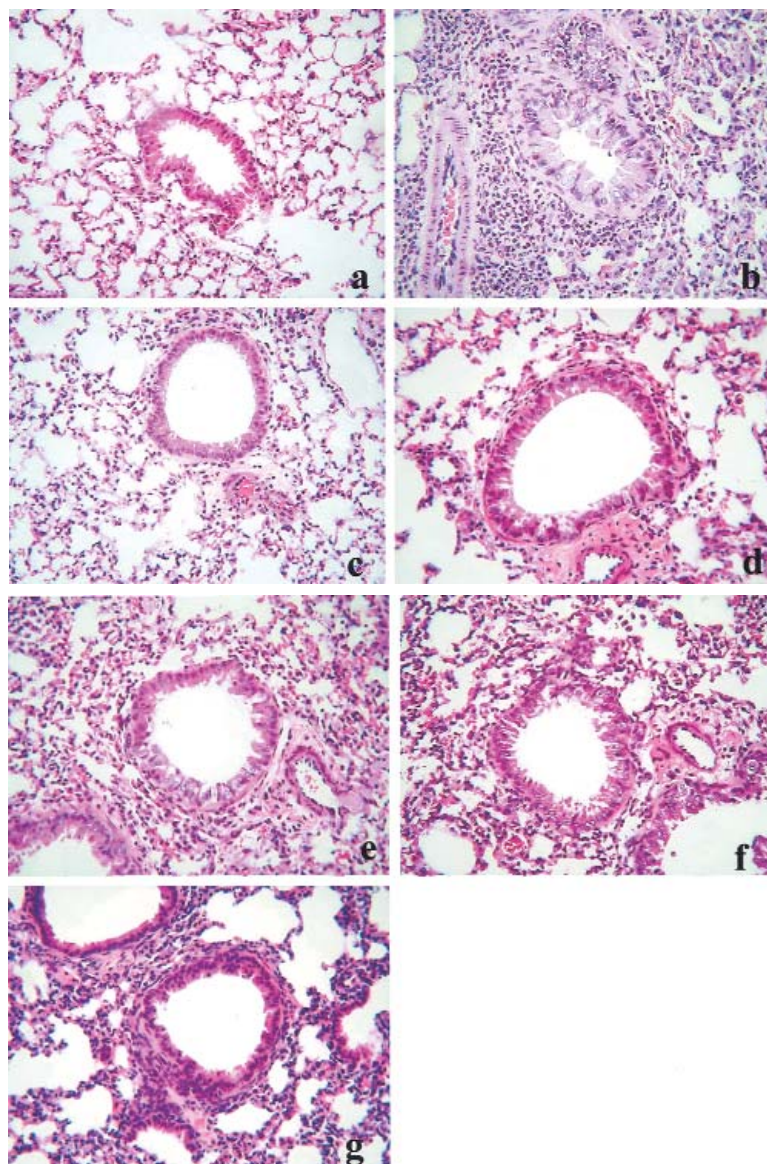


FIG 1. Histopathology. Representative specimens stained with hematoxylin-eosin are shown. **a**, Normal group. **b**, Sens group. **c**, Wk-S-Bud group. **d**, Daily-Bud group. **e**, Wk-C-Bud group. **f**, Wk-Empty-S group. **g**, Wk-Bud group. Original magnification $\times 100$.

noncompliance and treatment failures, which might result in increased hospitalizations and complications. This is the first study to investigate weekly therapy with budesonide encapsulated in stealth liposomes to treat experimental asthma. Our results show that stealth liposomes have a unique capacity to deliver budesonide effectively to the lungs, requiring only a fraction of the dosage and a less frequent dosing interval in comparison with conventional therapy.

The most important aim of the present study was to determine whether use of this drug delivery system alters the significant inflammatory airway response of asthma. Levels of immunologic markers implicated in the progression of asthma,²⁹⁻³² such as EPO activity in BAL, PB eosinophils, serum IgE levels, and lung inflammation as

seen on histologic examination, were decreased with this novel mode of drug delivery.

Our finding of worsening inflammation in 2 of the groups—Wk-Bud and Wk-C-Bud—was an unexpected finding. A possible mechanism is that Wk-Bud therapy or Wk-C-Bud therapy produces an initial rapid response followed by a rebound effect on inflammation. We were unable to find any previous reports on the effects of weekly therapy with budesonide. In addition, it has been shown that steroids encapsulated in conventional liposomes diffuse rapidly from these liposomes¹⁵⁻²⁰; it is thus possible that none of the budesonide encapsulated in the conventional liposomes resulted in sustained delivery to the lungs. Future studies by us, aimed at measuring the amount of budesonide that is being delivered to the lung

Histopathology Score

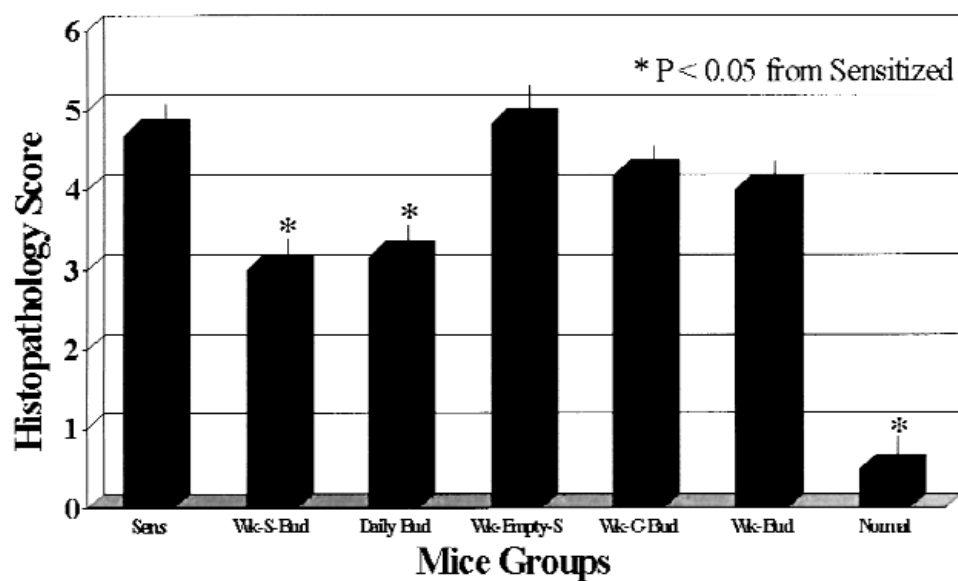


FIG 2. Total histopathology scores. Inflammation was categorized as none (score, 0), mild (score, 1-2), moderate (score, 3-4), or severe (score, 5-6). The score of each of the treatment groups was compared with the score of the Sens group.

Eosinophil Peroxidase Activity

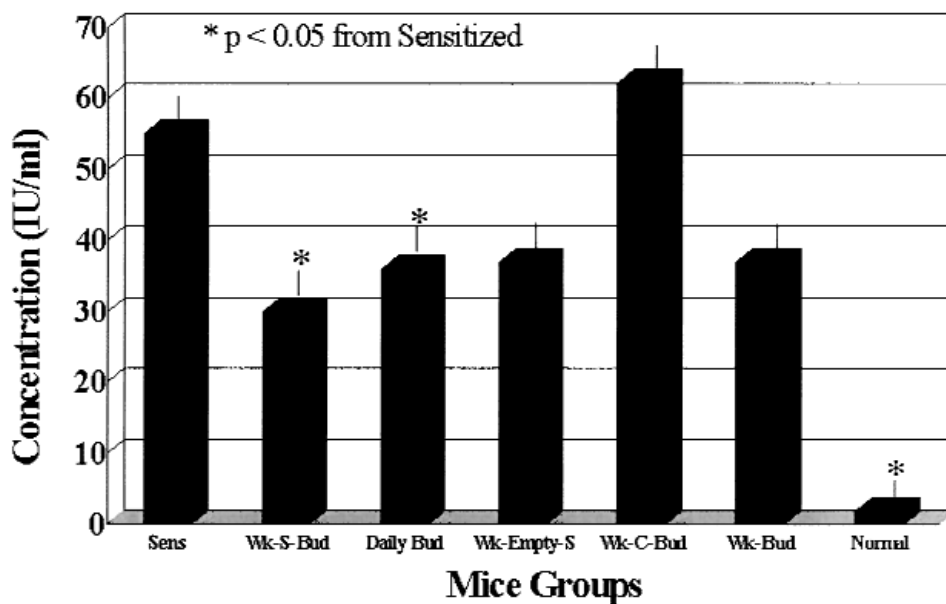


FIG 3. EPO activity in the BAL, expressed in international units per milliliter. The treated groups and the Sens group were compared with respect to EPO activity.

Peripheral Blood Eosinophils

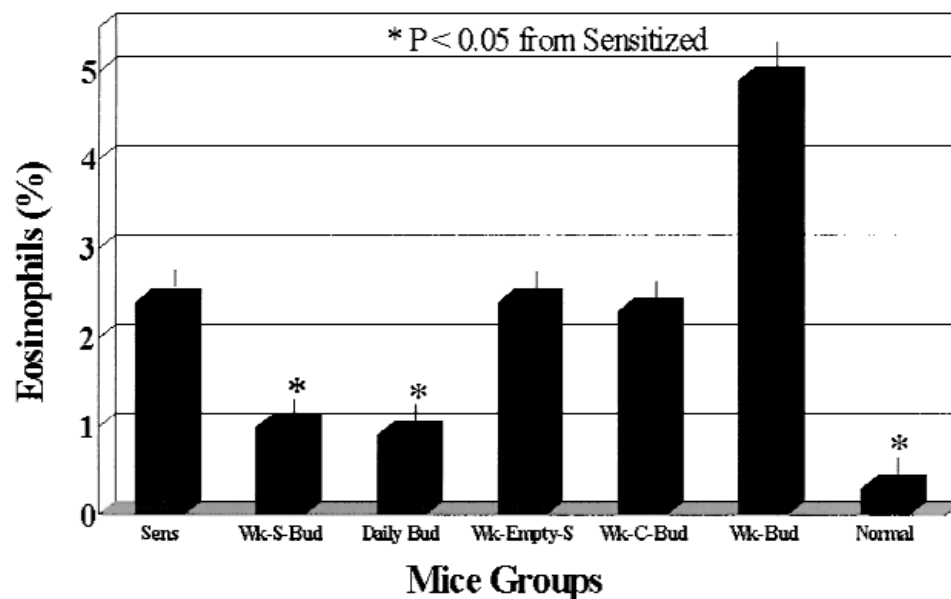


FIG 4. PB eosinophils. The treated groups and the Sens group were compared with respect to the percent of eosinophils per 100 white blood cells.

Serum IgE Levels

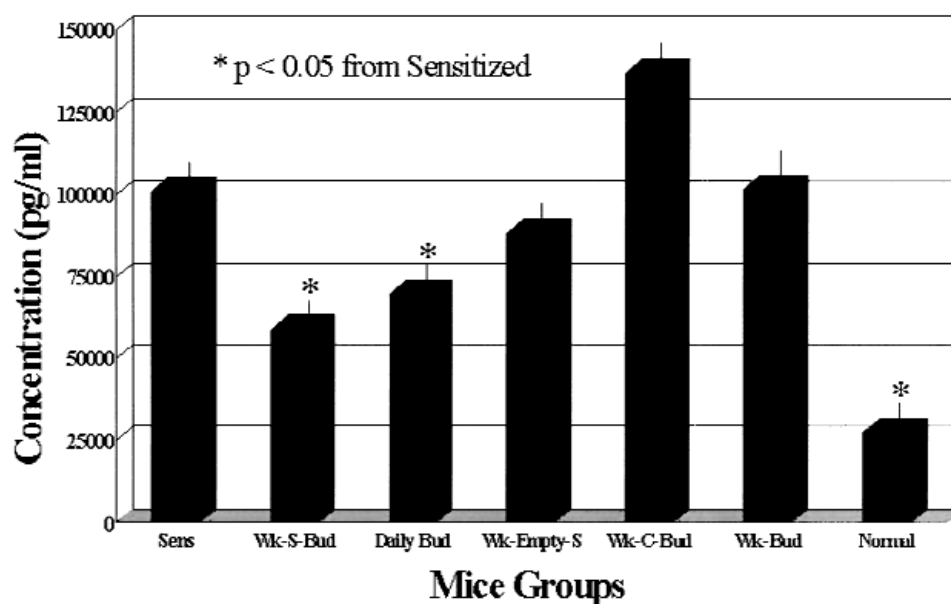


FIG 5. Serum IgE levels, expressed in picograms per milliliter. The treatment groups and the Sens group were compared with respect to IgE level.

and the amount remaining in conventional or stealth liposomes at the time of delivery, might aid in understanding the mechanisms involved.

The decrease in PB blood eosinophils seen in our study was consistent with previous reports that inhaled steroids reduce the production of pro-inflammatory cytokines, such

as IL-4 and IL-5 in BAL, which might lead to decreased bone marrow production or release of eosinophils.³³⁻³⁵ We also observed similar decreases in the levels of IL-4 and IL-5 in BAL and splenocyte culture supernatants, but only in the Wk-S-Bud and Daily-Bud groups.

We have shown that budesonide encapsulated in stealth liposomes significantly decreases inflammation in experimental asthma. Nevertheless, we questioned the safety of such a delivery system. We found that the animals tolerated the therapy without adverse side effects, such as abnormal weight gain, irritability, respiratory distress, and histologic abnormalities in the bone marrow, bone, spleen, liver, or gastrointestinal tract.

Encapsulation in stealth liposomes can thus be a safe and effective vehicle for delivery of inhaled steroids to the asthmatic lung. This unique drug delivery method might provide an alternative to daily budesonide therapy, with the potential to reduce toxicity and improve compliance for inhaled steroid therapy in asthma. Stealth liposome encapsulation might further be developed as a new method by which to deliver other drugs to the airways.

In memory of Dr P. R. J. Gangadharam (Ganga), who will be missed not only for his valuable contributions of nearly 50 years of research to drug-resistant tuberculosis but also for his support, guidance, and friendship.

We acknowledge the expertise of Dr Jordan N. Fink and his contribution in the preparation of the manuscript.

REFERENCES

- Weiss KB, Sullivan SD. The health economics of asthma and rhinitis. I. Assessing the economic impact. *J Allergy Clin Immunol* 2001;107:3-8.
- US Public Health Service. National Heart, Lung, and Blood Institute. Practical guide for the diagnosis and management of asthma. NIH publication No. 97-4053. Bethesda (MD): The Institute; 1997.
- Pedersen S, O'Byrne PM. A comparison of the efficacy and safety of inhaled corticosteroids in asthma. *Allergy* 1997;52:1-34.
- O'Byrne PM, Pedersen S. Measuring efficacy and safety of different inhaled corticosteroid preparations. *J Allergy Clin Immunol* 1998;102:879-86.
- Brown PH, Blundell G, Greening AP, Crompton GK. Screening for hypothalamic-pituitary-adrenal axis suppression in asthmatics taking high dose inhaled corticosteroids. *Respir Med* 1991;85:511-6.
- Wong J, Black P. Acute adrenal insufficiency associated with high dose inhaled steroids. *Br Med J* 1992;304:1415-6.
- Toogood JH, Sorva R, Puolijoki H. Review of the effects of inhaled steroids therapy on bone. *Int J Risk Safety Med* 1994;5:1-14.
- Bakker-Woudenberg IAJM, Lokerse AF, ten Kate MT, Monton JW, Woodle MC, Storm G. Liposomes with prolonged circulation and selective localization in *Klebsiella pneumoniae* infected lung tissue. *J Infect Dis* 1993;168:164-71.
- Gangadharam PRJ, Ashtekar DR, Flasher D, Düzgünes N. Therapy of *Mycobacterium avium* complex infection in beige mice with streptomycin encapsulated in sterically stabilized liposomes. *Antimicrob Agents Chemother* 1995;39:725-30.
- Düzgünes N. Treatment of human immunodeficiency virus, *Mycobacterium avium*, and *Mycobacterium tuberculosis* infections by liposome-encapsulated drugs. In: Lasic D, Papahadjopoulos D, editors. Medical applications of liposomes. Elsevier Science BV, Amsterdam, 1998; p. 189-219.
- Wasan KM, Lopez-Berestein G. The development of liposomal amphotericin B: an historical perspective. In: Lasic D, Papahadjopoulos D, editors. Medical applications of liposomes. Amsterdam: Elsevier Science BV; 1998; p. 165-80.
- Taylor KMG, Taylor G, Kellaway IW, Stevens J. The influence of liposomal encapsulation on sodium cromoglycate pharmacokinetics in man. *Pharm Res* 1989;6:633-6.
- Gonda I. Aerosols for delivery of therapeutic and diagnostic agents to the respiratory tract. *CRC Crit Rev Therap Drug Carrier Syst* 1990;6:272-313.
- Dijkstra J, Van Galen WJM, Hulstaert CE, Kalicharan D, Roerdink FH, Scherphof GL. Interaction of liposomes with Kupffer cells in vitro. *Exp Cell Res* 1984;150:161-76.
- Schreier H, Lukyanov AN, Hochhaus G, Gonzalez-Rothi RJ. Thermodynamic and kinetic aspects of the interaction of triamcinolone acetonide with liposomes. *Proc Intl Symp Cont Rel Bioact Mater* 1994;21:228-9.
- Gonzalez-Rothi RJ, Suarez S, Hochhaus G, Schreier H, Lukyanov A, Derendorf H, et al. Pulmonary targeting of liposomal triamcinolone acetonide. *Pharm Res* 1996;13:1699-703.
- Farr SJ, Kellaway IW, Carman-Meakin B. Comparison of solute partitioning and efflux of liposomes formed by a conventional and aerosolized method. *Int J Pharmaceut* 1989;51:39-46.
- Vidgren M, Waldrep JC, Arppe J, Black M, Rodarte JA, Cole W, et al. A study of ^{99m}technetium-labeled beclomethasone dipropionate dilauroylphosphatidylcholine liposome aerosol in normal volunteers. *Int J Pharmaceut* 1995;115:209-16.
- Waldrep JC, Gilbert BE, Knight CM, Black BM, Scherer P, Knight V, et al. Pulmonary delivery of beclomethasone liposome aerosol in volunteers. *Chest* 1997;111:316-23.
- Waldrep JC, Keyhani K, Black M, Knight V. Operating characteristics of 18 different continuous-flow jet nebulizers with beclomethasone dipropionate liposome aerosol. *Chest* 1994;105:106-10.
- Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc Natl Acad Sci* 1988;85:6949-53.
- Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivatives of poly (ethylene glycol) show prolonged circulation half-lives in vivo. *Biochim Biophys Acta* 1991;1066:29-36.
- Taylor KMG, Taylor G, Kellaway IW, Stevens J. The stability of liposome nebulization. *Int J Pharmaceut* 1990;58:57-61.
- Huang SK, Martin FJ, Friend DS, Papahadjopoulos D. Mechanism of stealth liposome accumulation in some pathological tissues. In: Lasic D, Martin F editors. *Stealth liposomes*. Boca raton (FL): CRC Press; 1995; p. 119-25.
- Busse WW, Lemanske RF Jr. Asthma. *N Engl J Med* 2001;344:350-62.
- Vignola AM, Chanez P, Campbell AM, Souques F, Lebel B, Enander I, et al. Airway inflammation in mild intermittent and in persistent asthma. *Am J Respir Crit Care Med* 1998;157:403-9.
- De Siqueria ALP, Russo M, Stell AA, Facincone S, Mariano M, Jancar S. A new murine model of pulmonary eosinophilic hypersensitivity: contribution to experimental asthma. *J Allergy Clin Immunol* 1997;100:383-8.
- Nandedkar SD, Konduri KS, Artwohl J, Bunte R, Gangadharam PRJ. A novel approach to ovalbumin sensitization in C57Bl/6 mice [abstract 580]. *J Allergy Clin Immunol* 2001;107:S176.
- Strath M, Warren DJ, Sanderson CJ. Detection of eosinophils using an eosinophil peroxidase assay. Its use as an assay for eosinophil differentiation factors. *J Immunol Methods* 1985;83:209-15.
- Mayer LB, Cullis PR, Bally MB. Designing therapeutically optimized liposomal anticancer delivery systems: lessons from conventional liposomes. In: Lasic D, Papahadjopoulos D, editors. *Medical applications of liposomes*. Amsterdam: Elsevier Science BV; 1998; p. 242-4.
- Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavamian N, Enander I, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033-9.
- Broide DH, Gleich GJ, Cuomo AJ, Coburn DA, Federman EC, Schwartz LB, et al. Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. *J Allergy Clin Immunol* 1991;88:637-48.
- Woolley MJ, Denburg JA, Ellis R, Dahlback M, O'Byrne PM. Allergen-induced changes in bone marrow progenitors and airway responsiveness in dogs and the effect of inhaled budesonide on these parameters. *Am J Respir Cell Mol Biol* 1994;11:600-6.
- Lonnkvist K, Hellman C, Lundahl J, Hallden G, Hedlin G. Eosinophil markers in blood, serum, and urine for monitoring the clinical course in childhood asthma: impact of budesonide treatment and withdrawal. *J Allergy Clin Immunol* 2001;107:812-7.
- Wardlaw AJ, Kay AB. The role of the eosinophil in the pathogenesis of asthma. *Allergy* 1987;42:321-35.