

Chemoprevention of Pulmonary Carcinogenesis by Aerosolized Budesonide in Female A/J Mice¹

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Abstract

This investigation is part of a continuing effort to develop effective chemoprevention for carcinogenesis of the lung. The present study explores the use of aerosol administrations for this purpose. The agent selected for initial study was the synthetic glucocorticoid budesonide. This selection was based on previous work in which budesonide added to the diet was found to inhibit pulmonary adenoma formation in female A/J mice. However, high dose levels were required, *i.e.*, of the order of 300 $\mu\text{g}/\text{kg}$, of body weight [L. W. Wattenberg and R. D. Estensen, *Carcinogenesis* (Lond.), 18: 2015–2017, 1997]. For aerosol administration of budesonide, a nose-only technique has been developed that entails nebulization of the compound dissolved in ethanol and subsequent stripping off of the solvent (less than 3 μl ethanol/liter of air remaining at the site of inhalation). The budesonide particles produced by the apparatus had a mass median aerodynamic diameter of less than 1 μm . An experiment has been carried out in which the inhibitory effects of aerosolized budesonide, given for 1 min six times a week, were studied. Concentrations of budesonide of 26, 81, and 148 $\mu\text{g}/\text{liter}$ of air (calculated doses of 23, 72, and 126 $\mu\text{g}/\text{kg}$ of body weight) were used. The aerosols were started 1 week after three oral administrations of benzo(a)pyrene (2 mg/20 g of body weight) to female A/J mice. All three doses of budesonide resulted in more than 80% inhibition of pulmonary tumor formation compared to the aerosol control and 90% or greater compared to mice not exposed to aerosol. The difference in inhibition is due to the aerosol procedure itself, which produces a reduction in tumor formation. A decrease in splenic weight (evidence of a systemic effect) occurred at all doses of budesonide. To the best of our knowledge, this is the first published effort at the use of aerosol administration to prevent neoplasia of the respiratory tract. The results of the present study show that administration of a potential chemopreventive agent by aerosol at a low dose can inhibit the occurrence of pulmonary carcinogenesis in female A/J mice.

Introduction

This investigation is part of a continuing effort to develop effective chemoprevention for carcinogenesis of the lung (1–4). The present study explores the respiratory delivery of aerosols for this purpose. Compounds under investigation are synthetic glucocorticoids. The choice of this group of compounds is based on previous work showing their efficacy when given by other routes of administration. A series of experiments has been carried out to determine the capacity of dietary additions of the synthetic glucocorticoid dexamethasone to prevent pulmonary tumor formation in female A/J mice. This com-

pound was found to inhibit both B(a)P⁴ and 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone-induced pulmonary carcinogenesis in these animals. Inhibition occurred when the administration of dexamethasone was limited to the postinitiation period, as well as when it was given throughout the entire carcinogenic process (4). In addition to the lung, dexamethasone also inhibits carcinogenesis of the forestomach and skin of the mouse when given in the postinitiation period (1, 5, 6). Dexamethasone has a number of biological effects that can account for its inhibitory properties (7–11). The capacity to mature type II alveolar cells, the major cell type occurring in pulmonary adenoma formation in the A/J mouse, is particularly noteworthy (7, 8). Which effect or combination of effects is responsible for its prevention of carcinogenesis of the lung, as well as in other tissues, has not been established.

The inhibitory effects of dietary administrations of dexamethasone on pulmonary carcinogenesis occurs only at high dose levels, *i.e.*, of the order of 0.5 mg/kg of diet (100 $\mu\text{g}/\text{kg}$ of body weight; Ref. 4). For acceptable use in human subjects as a chemopreventive agent, lower doses would be required. A possible means of obtaining inhibition at a lower dose level would be to administer the compound directly to the respiratory tract by aerosol. This form of administration delivers the inhibitor directly to the target tissue, which could result in lower doses being required and thereby reduced systemic toxicity. A considerable amount of research has been directed at identifying glucocorticoids with high topical potency in the lung and minimal systemic effects (12–14). These studies have been largely driven by the clinical use of chronic administration of such aerosolized glucocorticoids for the treatment of bronchial asthma (14). From this work, data have been accumulated that show that the synthetic glucocorticoid, budesonide, has favorable properties in this regard (14–16). At low dose levels, systemic effects on adrenal function are not demonstrable, but as the doses are increased, they are found (12).

Prior to its use in a respiratory delivery system, experiments were carried out to determine whether budesonide had efficacy in inhibiting pulmonary adenoma formation in A/J mice with dietary administration. Reduction of pulmonary adenoma formation did occur, but high dose levels were required, *i.e.*, 1.5 mg/kg of diet (300 $\mu\text{g}/\text{kg}$ of body weight; Ref. 17). This dose is approximately three times that of dexamethasone to produce the same degree of inhibition. These results are in accord with the anticipated lower systemic effects of the compound. With these data, it seemed feasible to undertake an investigation of the capacity of budesonide to inhibit pulmonary neoplasia in A/J mice when delivered to the respiratory tract as an aerosol.

To carry out these studies, an aerosol apparatus was required that had two essential features: (a) production of particle sizes that can reach the periphery of the lung and (b) delivery of concentrations of the test compound sufficient for efficacy under conditions of brief

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⁴ The abbreviations used are: B(a)P, benzo(a)pyrene; MMAD, mass median aerodynamic diameter.

aerosol exposures. In addition, a "nose-only" technique was desirable. An alternative is to use whole-animal exposure. This procedure has the serious defects of coating the animal fur with the test compound, resulting in oral consumption by licking as well as potential absorption through the skin. A nose-only aerosol delivery apparatus has been constructed that meets the necessary requirements. It entails the use of a conventional jet nebulizer for aerosol generation. The glucocorticoid is dissolved in ethanol, which subsequently is stripped away during passage through an aqueous trap. The budesonide particles produced have a MMAD of less than 1 μm , and the aerosol concentrations delivered are in the range necessary for efficacy. Using this apparatus, a high degree of inhibition of pulmonary tumor formation has been obtained.

Materials and Methods

Chemicals. The chemicals used were budesonide (>99% purity; Sigma Chemical Co., St. Louis, MO) and B(a)P (>98% purity; Aldrich Chemical Co., Milwaukee, WI).

Animal Experiments. Female A/J mice obtained from The Jackson Laboratories (Bar Harbor, ME) were used in all experiments. The animals were fed a semipurified diet consisting of 27% vitamin-free casein, 59% starch, 10% corn oil, 4% salt mix (USP XIV), and a complete mixture of vitamins (Teklad, Madison, WI). The mice were housed in a constant-temperature facility with controlled lighting; lights were turned on at 6 a.m. and off at 6 p.m. At 15 weeks of age, the mice were given the first of three administrations of 2 mg of B(a)P in 0.2 ml of cottonseed oil or cottonseed oil only (vehicle) by oral intubation. The time interval between the first and second doses was 4 days and between the second and third dose, it was 3 days. One week after the last administration of B(a)P, the mice were randomized by weight into groups of 15 animals each. The mice were reweighed at weekly intervals. Following randomization, the aerosol administrations were started. They were given for 1 min per day, 6 days per week for the duration of the protocol. The mice were exposed singly to the aerosol by placing their noses into the cone of the apparatus. Care was taken to handle the animals gently to minimize stress. The details of the aerosol apparatus and procedure are described below. The animals were sacrificed at the termination of the protocol, which was 16 weeks after the last dose of B(a)P. The mice were autopsied, and the lungs were taken for pulmonary adenoma counts using the procedure of Shimkin (18, 19), as previously described.

Aerosol Procedure. The aerosol apparatus consisted of a MiniHeart Nebulizer (Vortran Medical Technology Company, Sacramento, CA) held in an ice bath and connected by means of a flange fitting and T joint to a 375-mm Hopkins condenser (Corning). The condenser was heated by the water jacket at 37°C. The aerosol cloud then passed through a 105° adapter (Ace Glass) to which a 5-ml polypropylene pipet tip had been fitted. Eight mm of the tip were cut off, giving an open bore of about 2 mm, and this was immersed 2 inches deep in a 1-liter flask (Corning) filled with 800 ml of water. The flask and contents were heated to 25°C. The aerosol cloud was bubbled through the water in the flask and passed through the exit tube in the 105°C adapter and then through a 110-mm condenser, which was also heated to 37°C. After reheating, the aerosol was passed through a rubber hose to the nose cone. The nose cone was fabricated from a 6-ml disposable syringe casing (Monoject), from which 5 mm of the nose end were removed. Fourteen holes of about 2 mm in diameter were cut in the nose cone to allow exit of the aerosol after passing by the animal's nose.

For nebulization, budesonide was dissolved in ethanol and placed into the jet nebulizer. In these studies, the starting volume was 12 ml, and the nebulizer was chilled to 0°C in an ice bath 5 min prior to starting each run. The ethanol concentration was determined by gas chromatography at the apex of the nose cone, *i.e.*, the position at which the nose of the mouse would occupy the cone of the apparatus. The concentration was less than 3 μl of ethanol/liter of air. The nebulizer had an outflow gas flow rate of 1.5 liters/min when operated at a pressure of 20 p.s.i.g. With these conditions, the nebulizer had a total output rate of 290 μl of solution/min.

Budesonide Aerosol Concentration. The concentration of budesonide in the aerosol was determined by replacing the nose cone with a collection filter

assembly with Millipore filters. The aerosol was captured for a fixed time, and then budesonide was extracted from the filter. The concentration was determined spectrophotometrically using appropriate standards. The aerosol concentration was calculated as the mass collected in 1 min divided by the air flow rate.

Aerosol Particle Sizing. A (low flow rate) cascade impactor (InTox, Albuquerque, NM) was used to determine the size distribution of the aerosol budesonide particles. The MMAD and geometric SD were obtained from the cumulative undersized mass collected given as a function of logarithm of the cutoff diameter. For a solution concentration of 1 mg/ml, the MMAD was 0.83 μm with a geometric SD of 1.9. At the higher solution concentration, the MMAD was only slightly larger (<1.0 μm), with no change in polydispersity.

Monitoring Dose Delivery. The dose to the animal was estimated from the aerosol concentration, μg of budesonide per liter of air, as follows:

$$\text{Dose} = \frac{\text{Aerosol concentration} \times \text{RMV} \times \text{Exposure time}}{\text{Body weight}} \quad (\text{A})$$

where *RMV* is the respiratory minute volume, which was estimated with Guyton's formula (20); the exposure time was 1 min; and the body weight was taken to be 0.025 kg. The inherent assumption of this approach is that the inspired aerosol is completely deposited in the lung.

Statistical Analyses. Differences between groups in an experiment were examined by the nonparametric Kruskal-Wallis test because of inhomogeneous variances. If the overall test was significant, pairwise comparisons were carried out. The statistical package SAS was used.

Results

The results of the experiment to determine the efficacy of the respiratory delivery of budesonide by aerosol to inhibit B(a)P-induced pulmonary adenoma formation are shown in Table 1. All three concentrations of aerosolized budesonide showed >80% inhibition of pulmonary tumor formation when compared to the aerosol control group [B(a)P-Eth]. As discussed previously, the nebulization of ethanol does not result in any significant amount of the solvent reaching the nose cone (*i.e.*, <3 μl /liter). The data obtained with the experimental group in which water was nebulized in place of ethanol are virtually identical. An additional group of mice included in this study, B(a)P-C, was not exposed to the nebulization procedure. This group had a higher tumor count than the nebulization controls [B(a)P-Eth and B(a)P-H₂O]. Data from three groups of mice that did not receive B(a)P are shown in Table 1. A small number of pulmonary adenomas is found in this strain of mouse in the absence of carcinogen exposure.

In Table 1, data on body weight are recorded. The body weight of aerosol controls [B(a)P-Eth] and 1 mg of aerosolized budesonide [B(a)P-Bud (1 mg)] were virtually identical. The groups receiving the aerosolized budesonide at the 3- or 6-mg concentrations [B(a)P-Bud(3 mg) and B(a)P-Bud (6 mg)] showed lesser body weights, which were statistically significant. The body weights of mice that had not received aerosols were higher than those that had. Data on splenic weights also are provided in Table 1. All groups subjected to the aerosol procedure showed a decrease in splenic weight. This was greater for the animals receiving the budesonide than for the corresponding solvent controls.

Discussion

To the best of our knowledge, this is the first published effort at the use of aerosol administration to inhibit neoplasia of the respiratory tract. Respiratory delivery has the potential advantage of achieving high concentrations of the test agent at the target site with minimum systemic distribution. Thus far, no ideal glucocorticoid has been identified. Among the glucocorticoids, budesonide appears to have a

Table 1 Effects of budesonide delivered by aerosol on B(a)P-induced pulmonary adenoma formation in female A/J mice

Group designation	Carcinogen ^a	Aerosol ^b				Pulmonary adenomas		
		Budesonide (or vehicle) in nebulizer (mg/ml)	Budesonide in aerosol ($\mu\text{g}/\text{liter}$ of air)	Calculated dose of budesonide ^c ($\mu\text{g}/\text{kg}$ of body weight)	Final body weight ^d (g)	Splenic weight (mg)	No. of tumors per mouse	% inhibition ^e
B(a)P-C	+	None ^f			27	88	9.9 \pm 4.1 ^g	
B(a)P-H ₂ O	+	H ₂ O			23	57	5.0 \pm 2.4	
B(a)P-Eth	+	Ethanol (control)			22	59	4.7 \pm 3.3	
B(a)P-Bud (1 mg)	+	Budesonide (1 mg)	26	23	21	42	0.8 \pm 1.2 ^h	83
B(a)P-Bud (3 mg)	+	Budesonide (3 mg)	81	72	19	32	0.9 \pm 0.8 ^h	81
B(a)P-Bud (6 mg)	+	Budesonide (6 mg)	148	126	19	33	0.5 \pm 0.9 ^h	89
C	-	None ^f			27	85	1.2 \pm 1.4	
Eth	-	Ethanol (control)			23	63	0.7 \pm 0.8	
Bud (6 mg)	-	Budesonide (6 mg)	148	126	19	32	0.4 \pm 0.8	

^a Three administrations of 2 mg of B(a)P in 0.2 ml of cottonseed oil or cottonseed oil only (vehicle) were given by oral intubation to 15-week-old female A/J mice. The time interval between the first and second doses was 4 days, and between the second and third doses, it was 3 days. There were 15 mice/group.

^b Aerosol administrations were started 1 week after the last dose of B(a)P. They were given for 1 min, 6 days per week, for 16 weeks. Budesonide was dissolved in ethanol for aerosolization.

^c From Eq. A (see text).

^d Body weight at termination of the study. Average weights at the time of randomization 1 week prior to the start of aerosol administrations were 23 g.

^e Mean number of tumors in ethanol control group minus the number in the test group divided by the number in the ethanol control group \times 100.

^f No exposure to aerosol.

^g Mean \pm SD.

^h $P < 0.001$, budesonide versus ethanol control.

slightly more favorable profile than others in this regard (12). This is an area of considerable interest, because glucocorticoids and their analogues have been used for a considerable period of time in the treatment of bronchial asthma, and they now are possible candidates for the chemoprevention of cancer of the respiratory tract. With further research, compounds with increased favorable properties may be identified.

The present study demonstrates that budesonide, delivered as an aerosol, can inhibit pulmonary adenoma formation in female A/J mice. An area that merits discussion pertains to the reduced tumor count that results from mice receiving aerosols as contrasted to mice that have not been subjected to this treatment. Mice that are used in these experiments are kept in a protected environment with no known stress. Rooms are at constant temperature and humidity. The lighting is rigorously timed, and the animals have plentiful food and water. With this background, they are sensitive to various forms of handling for administration of test materials. In our own experience, we have had similar responses to those observed in the aerosol study in animals that received daily oral administration of preventive agents by gavage. Others have reported detectable stress responses due to i.v. administrations (21). Deliberate stressing of mice has been shown to cause a reduction of pulmonary adenoma formation (22). Ideally, it would have been desirable to have a null effect from handling. With the mice in the present study, even with committed, gentle handling, this did not occur. Nevertheless, the groups of mice treated with budesonide clearly had a marked reduction in pulmonary tumor formation as compared to controls subjected to the same experimental conditions. It might be possible to minimize or eliminate handling effects on pulmonary tumor formation by use of conditioning procedures (23, 24).

In Table 1, the splenic weight of the various experimental groups has been recorded. In mice, reduction in splenic weights is an index of the systemic effects of glucocorticoids. A reduction of splenic weight was found for all doses of the aerosolized budesonide used in the present study, and its magnitude was dose related. Thus, even at the lowest dose used, there was some evidence of a systemic effect. In clinical studies in humans with asthma treated with budesonide, dose levels of the order of 20 $\mu\text{g}/\text{kg}$ of body weight have been used. These dose levels are reported to produce little or no systemic effects (12, 14, 16). It remains to be determined whether reduction of pulmonary adenoma formation in the

mouse would occur at dose levels at which systemic effects are eliminated.

In summary, the present investigation represents a first effort at using an aerosol delivery system for chemoprevention of cancer of the respiratory tract. The merits of this approach in terms of the production of a high ratio of topical:systemic activity are evident. Glucocorticoids are a very active group of compounds in terms of their capacity to exert chemopreventive activity. This property makes them prime candidates for use with aerosol delivery technology. Identification of compounds of this category with an improved ratio of topical:systemic effects merits continued effort. The present study demonstrates that a chemopreventive agent delivered by an aerosol can inhibit pulmonary carcinogenesis in the mouse.

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