Dietary Phytoestrogens Have Anti-Inflammatory Activity in a Guinea Pig Model of Asthma (44504)

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Abstract. **Phytoestrogens are a normal constituent of soy protein and have been shown to have anti-inflammatory activity in various *in vitro* and *in vivo* models. The present study was designed to determine if a diet enriched in the phytoestrogen isoflavones, genistin and daidzin, would alter the antigen-induced cellular infiltration, particularly eosinophilia, characteristic of a guinea pig model of asthma. Throughout the duration of the study, guinea pigs were maintained on a control diet (standard guinea pig chow) or the same diet enriched in isoflavones. The animals were placed on the diet 2 weeks prior to active sensitization with ovalbumin (OA). Three weeks after sensitization, animals were challenged with OA aerosol. The cellular infiltration into the lung and protein and red blood cells (RBC) in the bronchoalveolar lavage fluid (BAL) were determined 17 hr later. In animals maintained on the control diet, OA aerosol challenge resulted in the expected increase in eosinophils in both the BAL and the lung tissue, an increase in neutrophils in the BAL, and an increase in protein and the number of RBC in the BAL. In contrast, in animals maintained on the isoflavone diet, the OA-induced eosinophilia in the lung tissue was significantly attenuated. In addition, OA challenge caused a greater increase in BAL protein in animals maintained on the isoflavone diet compared with animals on the control diet. Our results indicated that a diet enriched in isoflavones results in reduced antigen-induced eosinophilia in the lung in the guinea pig model of asthma. However, this beneficial anti-inflammatory effect of dietary phytoestrogens is accompanied by a potentially detrimental increase in antigen-induced leakage of protein into the airspace.**

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Phytoestrogens are naturally occurring plant products known to have estrogenic activity. One major chemical group of phytoestrogens is the isoflavonoids. Isoflavones occur predominantly as glycosides in plants. Daidzin and genistin are two isoflavone conjugates present in high amounts in soy. Cleavage by glucosidases in the intestine yields the aglycones daidzein and genistein. Ingestion of the isoflavones in modest amounts of soy products can result in circulating concentrations of phytoestrogens that exceed the amounts of endogenous estrogens (1, 2). The use of soy has significantly increased in recent years, as has the use of soy extracts as dietary supplements. In view of the lack of regulation of such extracts by the FDA, more studies are needed to ascertain both the positive and negative health effects of ingestion of isoflavones.

Considerable attention has been focused on phytoestrogens as being a potential benefit in the prevention of atherosclerosis or as anticancer agents (3, 4). Considerable literature also exists regarding the anti-inflammatory activities of the isoflavones. Isoflavones inhibit adhesion molecule expression (5–8), alter arachidonic acid metabolism (9), inhibit oxygen radical generation (10, 11), and inhibit chemotactic factor production (12). Nutrient intake can also influence cytokine production and reactive oxygen species, thus influencing antioxidant defense and the inflammatory process (13). Genistein, a principal soy isoflavone, is a tyrosine

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kinase inhibitor (14) with immunosuppressive and anti-inflammatory properties. The isoflavones have demonstrated anti-inflammatory potential in various animal models including chronic ileitis (15), inflammation-induced corneal neovascularization (16), and ischemia reperfusion injury (17). These anti-inflammatory activities suggest that the isoflavones may have potential utility in asthma where the inflammation, as evidenced by eosinophilic infiltration into the lung, is thought to be an important component of the pathology. Perhaps the use of soy extracts as a dietary supplement would be beneficial in reducing the chronic eosinophilic infiltration in the asthmatic lung. We hypothesized that a diet enriched in a soy extract containing the isoflavones genistin and daidzin may alter the cellular infiltration into the lung seen in an asthmatic response *in vivo*. Thus, we examined the ability of such a diet to affect cell infiltration into the lung and increased protein and red blood cells (RBC) in the airspace in a guinea pig model of asthma. Our results indicated that feeding a diet enriched in the isoflavones is anti-inflammatory and results in reduced antigen-induced eosinophilia in the lung. However, this beneficial effect is tempered by the realization that feeding a diet enriched in isoflavones also results in increased antigen-induced leakage of protein into the airspace, suggesting that other components of the immune-mediated inflammatory response are enhanced.

Materials and Methods

Diets. An extract was prepared from soybeans by Hauser Chemical Research, Inc. (Boulder, CO). The process for the isolation and purification of isoflavones is documented in U.S. Patent Number 5,679,806 (Oct 21, 1997). Briefly, an ethanol extract of soy molasses was applied to a reverse phase matrix (RPM) 1C column and isoflavone fractions collected and combined. The mixed isoflavones added to the diet had a combined purity of 34.6% (weight percentage) for the glycoside conjugates of the isoflavones, daidzin (18.8%) and genistin (15.8%). A standard vitamin-C–fortified, pelleted diet for guinea pigs was used (Teklad Guinea Pig Diet 7006, Harlan Teklad, Madison, WI). The isoflavone diet contained: Teklad Guinea Pig Diet 7006, ground, 996.3 g/kg; ascorbic acid, coated (97.5%), 1.7 g/kg; and isoflavone extract (soybean), 2.0 g/kg. The control diet contained: Teklad Guinea Pig Diet 7006, ground, 998.3 g/kg; and ascorbic acid, coated (97.5%), 1.7 g/kg. The diets were prepared by Harlan Teklad. The final pelleted diet was stored at 4°C until it was distributed to the food hopper of the cages. Animals were fed *ad libitum* with new food provided as needed every 2–3 days. Free access to tap water was provided throughout the study. The two diets were provided by Novartis Nutrition with a code, and the entire study was conducted in Duluth, MN without knowledge of which diet was the standard control diet or the isoflavone diet. The code was revealed after all experiments were completed and data were analyzed.

Experimental Design. Thirty-two female Hartley guinea pigs were obtained from Charles River Laboratories (Portage, MI). On arrival, the animals weighed between 165 and 206 g with an estimated age, based on their weight, of 2–3 weeks. On arrival (Day 0), guinea pigs were randomly assigned to a cage and housed in pairs throughout the study, with each pair of animals receiving the same diet for the duration of the study. Animals were weighed on arrival and three times a week thereafter. On Day 14, guinea pigs were actively sensitized by the intraperitoneal injection of 50 mg/kg OA in normal saline solution (NSS). Each cage was assigned to one of the following four treatment groups: i) isoflavone diet and OA aerosol; ii) isoflavone diet and NSS aerosol; iii) control diet and OA aerosol; and iv) control diet and NSS aerosol. Animals were challenged with aerosol on either Day 35 (n = 10), 37 (n = 10), or 39 (n = 12). For each challenge day, animals from three of the four groups were exposed to an aerosol. In this way, the eight animals from each treatment group were not all challenged on a single day. Thirty min before OA or NSS aerosol challenge, guinea pigs were pretreated intraperitoneally with the histamine H_1 receptor antagonist pyrilamine maleate (6.1 mg/kg) to prevent death from an acute anaphylactic reaction. Our previous studies have demonstrated the effectiveness and specificity of this dosage of pyrilamine (18). Animals were exposed to aerosol in pairs with either 1% OA solution or NSS for 5 min in a plexiglass chamber (22 × 22 × 29 cm) using a DeVilbiss Model 35B Ultrasonic Nebulizer as previously described (19). The aerosol output from the nebulizer was drawn through the aerosolization chamber using a vacuum pump at a flow of 8.5 l/min.

All treatment groups contained eight animals, except animals on the control diet challenged with OA aerosol where the number of animals was seven. During exposure of animals to OA aerosol, one animal died within a minute, gasping for air, suggesting that it was acute bronchoconstriction after the antigen challenge.

Measurement of Cellular Infiltration and Protein and RBC in the Bronchoalveolar Lavage (BAL). Seventeen hours after exposure to OA or NSS aerosol, guinea pigs were given a lethal dose of pentobarbital, and their lungs were lavaged as described previously (20). A total of 30 ml of room temperature phosphate–buffered saline (PBS) in 5-ml increments was used for lavage. The volume of BAL fluid recovered ranged from 27 to 28 ml and was no different in each of the four groups of animals as determined by ANOVA. The BAL was centrifuged to sediment the cells, and the BAL cell pellet was resuspended in 1.0 ml of PBS, pH 7.2 for determination of the total number of white blood cells (WBC) recovered in the BAL of each animal. The BAL supernatant did not have detectable amounts of hemoglobin as determined by absorbance at 412 nm. Total protein recovered in the BAL supernatant of each animal was determined by the method described by Lowry *et al.* (21). Lung lobes were removed and homogenized as previously described (20) for the de-

termination of neutrophil and eosinophil infiltration into the tissue.

Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) in the BAL cell suspension and lung homogenates were extracted, and activities were assayed as an indicator of the numbers of neutrophils and eosinophils, respectively. Our previous studies (20) have demonstrated that EPO and MPO are reliable estimates of the number of eosinophils and neutrophils. We have reported that MPO measurements correlated with the number of neutrophils (r^2 = 0.81) but did not correlate strongly with the number of eosinophils (r^2 = 0.05). In addition, the EPO activity correlated with the number of eosinophils (r^2 = 0.79), but not well with the number of neutrophils (r^2 = 0.09). The MPO assay measures enzyme activity spectrophotometrically in the presence of o-dianisidine hydrochloride with 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO) in 50 m*M* KH_2PO_4, pH 6.0. For the BAL, the activity of the whole lung lavage is expressed as total units MPO in the recovered lavage fluid. For the right caudal lobe, MPO activity is expressed as the units MPO/g dry weight. The EPO assay measures enzyme activity spectrophotometrically in the presence of o-phenylenediamine dihydrochloride in PBS with 2% Triton (Sigma Chemical Co., St. Louis, MO) in Tris buffer, pH 8.0. In the BAL, the EPO activity is expressed as the total OD/min in the recovered lavage fluid. For the left caudal lobe, the EPO activity is expressed as OD/min/g dry weight. To insure that the isoflavones were not affecting the EPO and MPO measurements, the effects of dimethylsulfoxide (DMSO) and genistein dissolved in DMSO were determined. Inclusion of 13 μm genistein in the cuvette during the assay of EPO or MPO did not significantly alter the enzyme activity.

BAL cells from each animal were resuspended to 1 ml with PBS. The BAL cell suspension was sonicated to release hemoglobin followed by centrifugation. The absorbance of this supernatant at 412 nm was used as an estimate of the total number of RBC recovered in the BAL (i.e., OA-induced hemorrhage into the airspace) (20, 22).

Enzyme-Linked Immunosorbent Assay (ELISA) for OA-Specific Guinea Pig IgG1. This method is based on the study of Kawabata *et al.* (23) for the optimization and validation of an ELISA to measure antigen-specific guinea pig IgG1 antibody and our previous studies measuring trimellitic anhydride-specific IgG1 in the serum of guinea pigs (24). The ELISA assay was conducted as previously described for trimellitic anhydride-specific IgG1 except OA was used as the antigen coating the ELISA plates. Concentrations of OA used to coat the plate, as well as amounts of primary and secondary antibodies used in the ELISA were determined previously in our laboratory to be optimal and to reflect relative concentrations of OA-specific antibody. The relative concentration of OA-specific IgG1 was determined as previously described (24). The data are expressed as the concentration of OA-specific IgG1 in the sample divided by the concentration of OA-specific IgG1 in the standard, defined as 1. The IgG standard was prepared by passing a pool of serum from OA-sensitized guinea pigs over a Protein A Sepharose column. The recovered IgG was dialyzed against NSS and aliquoted for use as a standard in the assay.

Statistical Analysis. For all data, values were log-transformed to equalize the variances. Values in all figures represent the mean ± SE of determinations in eight different animals, with the exception of the group receiving the control diet and challenged with OA (n = 7). ANOVA with preplanned comparisons was used to generate two-tailed P-values. $P < 0.05$ was considered significant. The four groups of animals considered for comparison were the following: i) isoflavone diet and OA aerosol; ii) isoflavone diet and NSS aerosol; iii) control diet and OA aerosol; and iv) control diet and NSS aerosol. The comparisons of relevance for each measured variable were the following: NSS aerosol in the control versus the isoflavone diet was compared to determine if the diet changed the baseline values for each of the variables; and OA aerosol in the control versus the isoflavone diet were compared to determine if the OA-induced response was affected by the diet.

Results

Animal Weight. Animals were weighed every 2–3 days throughout the course of the study. On arrival, the animal weight range was 165–206 g. The average weight of the animals in each of the four experimental groups (isoflavone diet and OA aerosol, isoflavone diet and NSS aerosol, control diet and OA aerosol, control diet and NSS aerosol) was compared at time of sensitization, time of aerosol challenge, and time of lavage. At each time point, there was no significant difference in the animal weight for each of the four groups.

Effect of Isoflavone Diet on OA-Induced Cellular Infiltration into the BAL. Cellular infiltration was evaluated 17 hr after OA challenge. The volume of BAL fluid recovered ranged from 27 to 28 ml and was no different in each of the four groups of animals as determined by ANOVA. The cellular content of the BAL was assessed by counting the total number of WBC recovered, as well as by analyzing the EPO and MPO activity of the cell pellet as an indicator of the number of eosinophils and neutrophils, respectively. For each of the diets, challenge with OA aerosol caused a significant increase in the WBC, EPO, and MPO in the BAL compared with the NSS aerosol (Fig. 1). If the NSS aerosol was compared for each diet, no differences were detected (i.e., the baseline number of cells was the same in both the control diet and isoflavone enriched diet after NSS aerosol). In addition, the diet did not affect the magnitude of the OA-induced increase in WBC, EPO, and MPO into the BAL (isoflavone diet and OA aerosol compared with the control diet and OA aerosol).

Effect of Isoflavone Diet on OA-Induced Cellular Infiltration into the Lung. Eosinophil infiltration into the lung tissue was assessed by determining the EPO

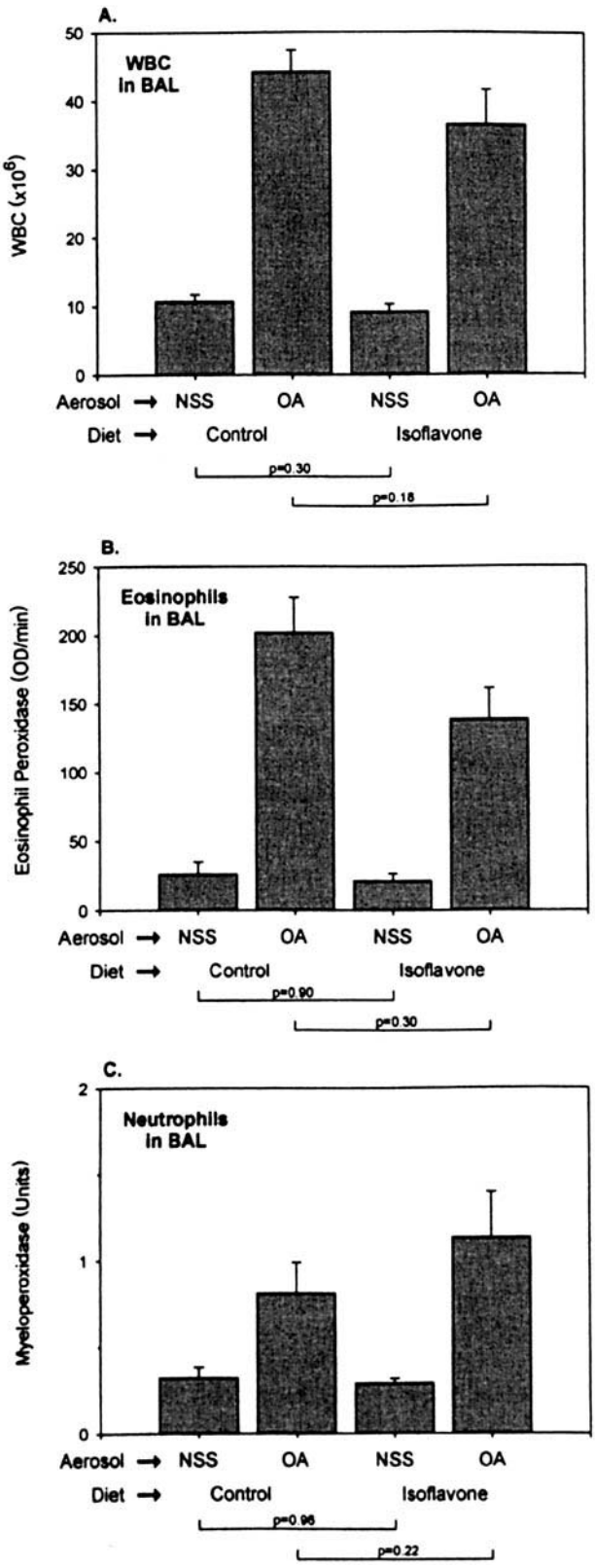

Figure 1. The effect of challenge with NSS or OA aerosol on the number of cells recovered in the BAL of guinea pigs maintained on either the control or isoflavone diet. Values represent the mean ± SE of experiments in seven to eight animals. *P*-values are shown for each of the preplanned comparisons. (A) Total number of WBC. (B) EPO activity as a measure of the number of eosinophils. (C) MPO activity as a measure of the number of neutrophils.

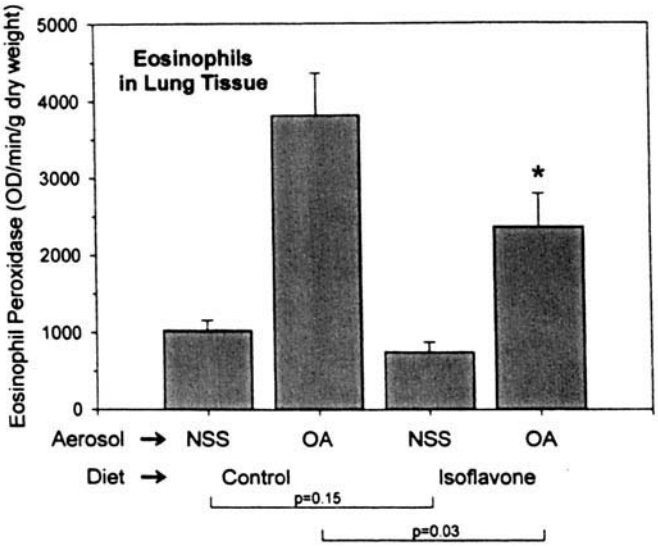

Figure 2. The effect of challenge with NSS or OA aerosol on the EPO activity of the lung tissue from guinea pigs maintained on either the control or isoflavone diet. Values represent the mean ± SE of experiments in seven to eight animals. *P*-values are shown for each of the preplanned comparisons. *Significantly different ($P < 0.05$) when OA aerosol is compared between the control diet and isoflavone diet.

content of the lung tissue. As seen in Figure 2, OA challenge resulted in an increase in the EPO in the lung tissue for each of the diets compared with their respective NSS aerosol control. The baseline EPO was the same in guinea pigs fed the control diet or isoflavone-enriched diet after NSS aerosol. However, the OA-induced eosinophil infiltration was significantly different when comparing the two diets, suggesting that eosinophil infiltration into the lung was reduced in animals on the isoflavone diet.

Neutrophil infiltration was determined by measuring the MPO content of the lung tissue. In contrast to the EPO in lung, OA challenge did not result in a significant increase in MPO in the lung tissue (Fig. 3). The MPO content of the lung tissue was the same in guinea pigs fed the control or isoflavone diet after either NSS or OA aerosol challenge.

Effect of Isoflavone Diet on OA-Induced Increases in Protein and RBC in the BAL. The total amount of protein recovered in the BAL was determined for each animal. As seen in Figure 4, the isoflavone diet did not affect the baseline level of protein significantly in the BAL of animals receiving NSS aerosol. OA challenge resulted in an increase in BAL protein for each of the diets. However, the protein accumulation in the BAL after OA challenge was significantly different when comparing the two diets, suggesting that the isoflavone diet enhanced the leakage of protein induced by OA.

The total number of RBC recovered in the BAL was assessed for each animal by lysing the cell pellet and determining the quantity of hemoglobin released (OD at 412 nm). OA challenge also resulted in an increase in the number of RBC in the BAL for each of the diets (Fig. 5). The

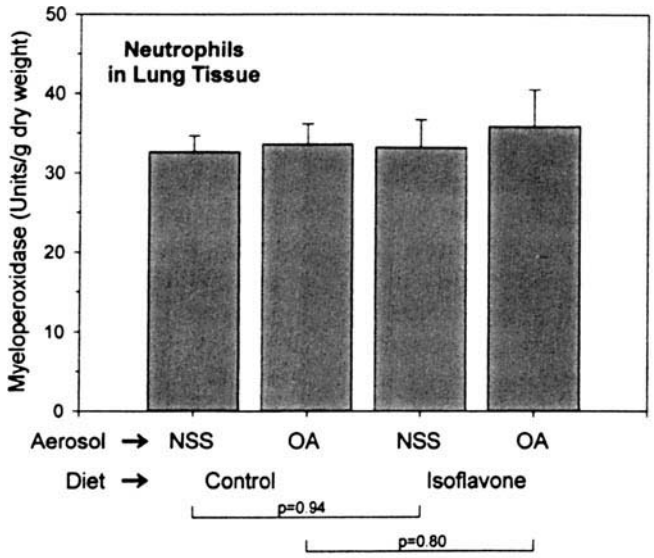

Figure 3. The effect of challenge with NSS or OA aerosol on the MPO activity of the lung tissue from guinea pigs maintained on either the control or isoflavone diet. Values represent the mean ± SE of experiments in seven to eight animals. *P*-values are shown for each of the preplanned comparisons.

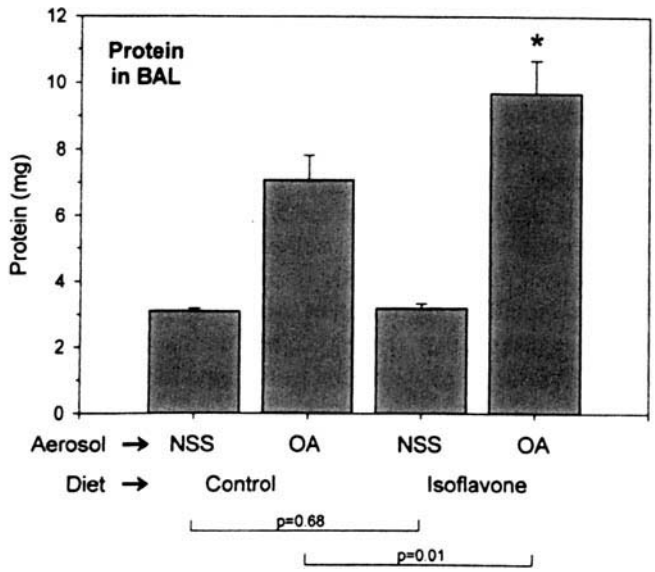

Figure 4. The effect of challenge with NSS or OA aerosol on the total protein content of the BAL supernatant recovered from guinea pigs maintained on either the control or isoflavone diet. Values represent the mean ± SE of experiments in seven to eight animals. *P*-values are shown for each of the preplanned comparisons. *Significantly different ($P < 0.05$) when OA aerosol is compared between the control diet and isoflavone diet.

number of RBC in the BAL after NSS aerosol was the same with either diet. Similarly, the number of RBC in the BAL after OA challenge was the same for both diets.

Effect of Isoflavone Diet on OA-Specific IgG1. OA-specific IgG1 in the serum was determined for each of the four treatment groups at the time cellular infiltration was measured. The means for the four treatment groups ranged from 0.10 to 0.24 (data not shown). The data are expressed as the concentration of OA-specific IgG1 in the sample divided by the concentration of OA-specific IgG1 in the

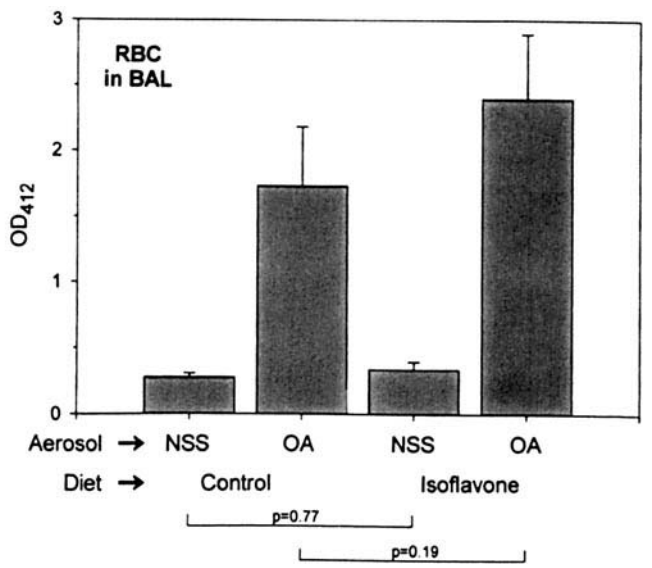

Figure 5. The effect of challenge with NSS or OA aerosol on the number of RBC recovered in the BAL of guinea pigs maintained on either the control or isoflavone diet. Values represent the mean ± SE of experiments in seven to eight animals. *P*-values are shown for each of the preplanned comparisons. Absorbance at 412 nm (OD_{412}) of the lyzed cell pellet from the BAL was used as an estimate of the number of RBC in the BAL.

standard, which is defined as 1. ANOVA of the log-transformed data revealed that there was no significant difference between the four treatment groups.

Discussion

Asthma is defined as a reversible airway obstruction associated with inflammation and airway hyperresponsiveness. For undefined reasons, the incidence of asthma is on the rise with a documented increase in morbidity and mortality, especially in pediatric populations (25). Treatment of asthma has changed from simply treating the bronchoconstriction with bronchodilators to treating the inflammatory response with inhaled anti-inflammatory drugs (26). Important components of asthma include bronchoconstriction, increased airway microvascular permeability, increased mucous production, cellular infiltration into the lung (particularly eosinophils), and airway hyperresponsiveness.

In the guinea pig, the lung is the target organ for allergen challenge, making it a useful model of asthma. Aerosol antigen challenge of a sensitized animal is known to cause an increase in the number of eosinophils in the BAL as well as in the airway wall (19, 27). Our results indicated that a diet enriched in isoflavones results in reduced antigen-induced eosinophilia in the lung, a desired therapeutic outcome.

Antigen challenge of the lung is also known to cause other components of the inflammatory response such as increased microvascular permeability to protein. This change allows the passage of plasma proteins, but not RBC, out of the vasculature and into the extravascular space and/or airspace. A more severe reaction to antigen challenge may lead to a breach of the normal barrier and an increase

in RBC in the airspace, or hemorrhage into the airspace occurs. This antigen-induced hemorrhage into the airspace can be accompanied by greater amounts of plasma protein entering the airspace. In our model of asthma, some injury occurred as shown by RBC in the BAL after aerosol OA challenge (19). The amount of protein in the airspace also increased after antigen challenge and could be due to increased microvascular leakage and/or hemorrhage. In animals fed the diet enriched in isoflavones, the amount of protein in the airspace was greater than in the animals fed the standard diet. This suggests that the potentially beneficial effect of dietary phytoestrogens in reducing eosinophilia has to be balanced with the knowledge that protein in the airspace increased, suggesting that other components of the inflammatory response are enhanced by a diet enriched in isoflavones.

How much soy extract did these guinea pigs ingest? Because of the nature of the study design, we can only estimate the maximal amount of feed these animals ingested over the course of the study. We weighed the amount of food added to the hopper as well as the amount of food remaining when the hopper was refilled. Such a measurement does not account for food that was dropped in the bottom of the cage while the animals were feeding. Animals were housed in pairs, so assuming each of the animals in the cage ate an equivalent amount of food, the average daily food consumption by each guinea pig over the course of the study was maximally 29–30 g/guinea pig/day. This would represent an estimate of maximal consumption of ≈ 40–42 mg isoflavone/kg/day for each guinea pig. In a human, estimates of ≈ 0.7 mg/kg/day of total isoflavones is considered moderate consumption (2). Determination of blood levels of the phytoestrogens was beyond the scope of this study. However, in view of the effects of the isoflavonoids on the asthmatic response in the guinea pig, more studies are warranted to define the amounts of isoflavones that would produce the observed effects.

Limited studies have been done examining the effect of diet on cellular infiltration into the asthmatic lung. A recent study published in abstract form (28) examined the effect of four different flavonoid compounds, including genistein, on the OA-induced eosinophilia in a mouse model of asthma. This study indicated that the flavonoids inhibited cytokine production from cells *in vitro*, but were ineffective in protecting against allergen-induced eosinophil influx. In a related asthma model, Wong *et al.* (29) found that genistein inhibited OA-induced contraction of isolated guinea pig bronchi, suggesting that the asthmatic bronchoconstriction might be reduced by isoflavone administration. In addition, he found that genistein and other tyrosine kinase inhibitors reduced antigen-induced release of histamine and peptidoleukotrienes from lung fragments *in vitro*. Mediators released immediately after antigen challenge may be responsible for setting in motion the events that lead to eosinophil infiltration. For example, studies of Underwood *et al.* (30) have demonstrated that aerosol administration of LTD_4 leads to a persistent eosinophilia in the guinea pig up to 4 weeks later. Thus, a reduction in leukotriene release at the time of antigen challenge may result in a reduced eosinophilia 24 hr later. Further studies *in vivo* would be necessary to test this possibility.

In the mouse model of asthma, each of the isoflavones was administered separately at a defined dose (28). In our study, isoflavones were provided as a soy extract incorporated into the diet to more closely mimic the administration of a diet high in soy. Certainly, the component of the isoflavone extract responsible for the changes in cellular infiltration and protein in the airspace is unknown, and no mechanism of action can be inferred from this study. The animals gained equivalent weight on either diet. In addition, our measurement of OA-specific IgG1 antibody in response to sensitization with OA, indicated that regardless of diet, the animals responded to antigen with an equivalent production of antibody. Thus, any differences seen in the cellular infiltration cannot be attributed to differences in the degree of sensitization of the animals.

In our study, isoflavones were administered in the diet for a total of 5 weeks; 2 weeks before and 3 weeks after sensitization. Guinea pigs, weaned at ≈ 1 week of age, were obtained from the supplier at ≈ 2–3 weeks of age. Thus, the guinea pigs used in our study spent 5 weeks of their 7- or 8-week life span on the test diet. Whether this length of time is necessary to see the changes in eosinophilia or protein in the BAL is not known and should be examined in the future. In a mouse model of asthma (28), animals were fed isoflavonoids for 3 weeks commencing at the time of sensitization. Changes in eosinophilia were not noted after antigen challenge following a 3-week sensitization period. Thus, it may be necessary to be exposed to isoflavones for a longer period of time, or for a sufficient period of time prior to sensitization, for an anti-inflammatory effect to be seen.

Eosinophil trafficking is an area of intense interest in asthma and allergic diseases (31). Recent studies in a mouse model of asthma (32) have suggested that eosinophils in the lung interstitium rather than in the airway lumen are the important cells in determining whether airway hyperresponsiveness develops. Thus, cells in both the lung homogenate (interstitium) and the airspace were monitored in our study. The final destination of the inflammatory cells in either the tissue or airspace, depends on the nature, intensity, and location of the chemotactic stimuli, as well as the time course of the response. Our previous studies (19) in this model of guinea pig asthma have shown that 6 hr after OA aerosol challenge, neutrophils and eosinophils are increased in both the BAL and lung homogenate. Twenty hours after OA aerosol, the number of eosinophils are still elevated. However, neutrophils in the lung homogenate, but not BAL, return to control 20 hr after OA aerosol.

The potential anti-inflammatory benefit of the isoflavone diet in the guinea pig model of asthma needs to be balanced by the possibility that such dietary modifications may also be detrimental. Certainly, one can only speculate

as to the active component(s) in the diet. In addition, multiple mechanisms can be hypothesized regarding how the diet enriched in isoflavone can have such an effect on the asthmatic response *in vivo*. However, further study is required to determine the active component(s) of such an isoflavone extract as well as to determine the mechanism of the reduced eosinophilia or the increased protein in the BAL. The prevalence of asthmatics in the population and the ready availability of soy extracts would suggest that both the potentially beneficial as well as detrimental effects of such dietary supplements should be thoroughly investigated.

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