HHS Public Access

Author manuscript

J Immunotoxicol. Author manuscript; available in PMC 2015 November 19.

Published in final edited form as:

J Immunotoxicol. 2014 ; 11(3): 268–272. doi:10.3109/1547691X.2013.843620.

Immune stimulation following dermal exposure to unsintered indium tin oxide

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Abstract

In recent years, several types of pulmonary pathology, including alveolar proteinosis, fibrosis, and emphysema, have been reported in workers in the indium industry. To date, there remains no clear understanding of the underlying mechanism(s). Pulmonary toxicity studies in rats and mice have demonstrated the development of mediastinal lymph node hyperplasia and granulomas of mediastinal lymph nodes and bronchus-associated lymphoid tissues following exposure to indium tin oxide. Given the association between exposure to other metals and the development of immune-mediated diseases, these studies were undertaken to begin to investigate the immuno-modulatory potential of unsintered indium tin oxide (uITO) in a mouse model. Using modifications of the local lymph node assay, BALB/c mice (five animals/group) were exposed topically via intact or breached skin or injected intradermally at the base of the ear pinnae with either vehicle or increasing concentrations 2.5–10% uITO (90:10 indium oxide/tin oxide, particle size <50 nm). Dose-responsive increases in lymphocyte proliferation were observed with a calculated EC3 of 4.7% for the intact skin study. Phenotypic analysis of draining lymph node cells following intradermal injection with 5% uITO yielded a profile consistent with a T-cell-mediated response. These studies demonstrate the potential for uITO to induce sensitization and using lymphocyte proliferation as a biomarker of exposure, and demonstrate the potential for uITO to penetrate both intact and breached skin.

Keywords

Immune stimulation; immunotoxicity; unsintered indium tin oxide

Introduction

Numerous metals are known to induce hypersensitivity responses resulting in dermal, respiratory, and systemic diseases (Table 1). Occupational exposure to these metals results

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Declaration of interest

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

in varying levels of morbidity and mortality from chronic allergic contact dermatitis to potentially fatal pulmonary disease or anaphylaxis. Recently, attention has been drawn to the occurrence of a rare pulmonary disease, characterized by severe alveolar proteinosis, seen in workers exposed to indium-tin oxide (ITO). Indium is a rare metal recovered for commercial use via extraction from other mined metals, most often zinc. It is refined primarily in China, Japan, the Republic of Korea, and Canada (POLINARES, 2012). The predominant use of indium is in the production of ITO. Because of its electrical conductive properties, thin-film coatings of ITO have historically been used in the production of solar panels, solders, alloys, and semiconductors. More recently, ITO usage has increased with the popularity of touch-screen computer and liquid crystal display technologies, which also use the electrically conductive and light transparent ITO coatings (U.S. Geological Survey, 2013). Potential worker exposure to indium and indium compounds occurs during mining, production, and reclamation processes.

In addition to pulmonary alveolar proteinosis, other pathologies including pulmonary fibrosis, emphysema, and granulomas with associated cholesterol clefts have been diagnosed in indiumexposed workers in the US, China, and Japan (Cummings et. al., 2012). Although a possible mechanism related to autoimmuneinduced dysfunction of macrophages has been proposed (Cummings et al., 2010), the pathophysiology and natural history of indium-induced pulmonary disease(s) have not been elucidated. Given the potential of other metals to induce hypersensitivity responses and the lack of a complete understanding of the pathophysiology of the observed pulmonary disease in ITO-exposed workers, these animal studies were undertaken to evaluate the potential of unsintered ITO (uITO) to induce immune stimulation.

Materials and methods

Animals

Female BALB/c mice were purchased from Taconic Farms (Germantown, NY) at 6–8 weeks-of-age and were allowed to acclimate for a minimum of 5 days before they were randomly assigned to treatment groups. Animals were weighed and individually identified via tail markings using a permanent marker, and an analysis of variance on body weights was performed to ensure homogeneous distribution of animals across treatment groups. Mice were housed five per cage in ventilated plastic shoebox cages with hardwood chip bedding, and provided *ad libitum* access to NIH-31 modified 6% irradiated rodent diet (Harlan Teklad, Frederick, MD) and tap water from water bottles. The temperature in the animal facility was maintained at 68–72°F and the relative humidity at 36–57%. The light/dark cycle was maintained on 12 h intervals. All animal experiments were performed in the Association for Assessment and Accreditation of Laboratory Animal Care International accredited National Institute for Occupational Safety and Health animal facility in accordance with an animal protocol approved by the Institutional Animal Care and Use Committee.

Chemicals

Unsintered indium tin oxide, < 50 nm (CAS# 50926-11-9), vehicles including dimethyl sulfoxide (DMSO, CAS # 67-68-5) and USP grade phosphate-buffered saline (PBS, pH 7.4), as well as a-hexylcinnamaldehyde (HCA, CAS# 101-86-0) positive control were purchased from Sigma Aldrich (St. Louis, MO).

Toxicity study

An initial toxicity study was conducted to determine the test article concentrations to be used in the subsequent studies. Three animals were exposed topically to 10% uITO that was chosen based on the highest concentration that remained in suspension following 10 min of sonication. To increase bioavailability, each day before dosing and following cleaning the ear with 70% ethanol, the dorsal aspect of each ear pinna was breached using a Multi-Test II device (manufactured by Lincoln Diagnostics, Inc, Decatur, IL). This device is commonly used in clinical practice for allergy skin testing. Animals were then exposed on each ear to 25 μl of vehicle (DMSO) or 10% uITO for three consecutive days. Dosing suspensions were vortexed for ≈5 s before being withdrawn from the dosing vial for exposures. Animals were observed daily for systemic signs of toxicity (ruffled fur, abnormal posture, isolation in the cage, abnormal exudates from eyes, nose, anus) and for local signs of irritation including erythema and swelling. Animals were weighed the day prior to the first exposure and on Day 6 prior to euthanasia.

Modified local lymph node assay

To determine the potential for uITO to induce immune stimulation, local lymph node assays including modifications to routes of exposure were conducted following topical exposure via intact or breached skin or following intradermal injection. Given that there was concern for the potential of uITO to penetrate intact skin, the initial study consisted of dermal exposure on the dorsal surface of each ear (25 μl per ear) to groups of animals with either intact or breached skin. Breaching was accomplished using a Multi-Test II device as described above. To increase bioavailability, additional studies were conducted following 10 μl intradermal injections (using a 26-guage needle) of test article or PBS vehicle at the base of the ear pinna bilaterally. Based on the results of the toxicity study and the limits of uITO to go into suspension, dose response studies were conducted with concentrations of 2.5%, 5%, and 10% (w/v) uITO. Five animals per group were used for all studies.

Following exposures, the LLNA was performed according to the method described in the Interagency Coordinating Committee on the Validation of Alternative Methods Peer Review Panel report (NIEHS, 2010) with minor modifications. Briefly, mice (five per group) were exposed to vehicle, increasing concentrations of uITO, or positive control (30% HCA, for intact dermal exposure study) for three consecutive days. Animals were allowed to rest for 2 days following the last exposure. On Day 6, mice were injected intravenously via the lateral tail vein with 20 μCi [^{3}H]-thymidine (specific activity 2 Ci/mmol; Dupont NEN, Boston, MA). Five hours after thymidine injection, mice were euthanized via CO_2 asphyxiation, and the left and right cervical draining lymph nodes (DLN located at bifurcation of jugular vein) were excised and pooled for each animal. Single cell suspensions were made and incubated overnight in 5% trichloroacetic acid and samples were counted using a Packard Tri-Carb

2500TR liquid scintillation analyzer. Stimulation indices (SI) were calculated by dividing the mean disintegrations per minute (DPM) per test group by the mean DPM for the vehicle control group. EC3 values (concentration of chemical required to induce 3-fold increase over vehicle control) were calculated based on the equation from Basketter et al. (1999).

Phenotypic analysis of draining lymph node cells following intradermal exposure

To further evaluate the immune response to uITO exposure, phenotypic analysis of DLN was conducted using flow cytometry as previously described with minor modifications (Franko et al., 2012). Mice (10 per group) were injected intradermally with PBS or 5% uITO at the base of each ear (10 μl per ear) for three consecutive days. Five animals per group were allowed to rest for 2 days and euthanized by CO_2 inhalation on Day 6 to coincide with the time course of the lymphocyte proliferation assay and the remaining five animals per group were allowed to rest for 7 days after the final exposure and then euthanized on Day 10 by CO_2 inhalation. Day 10 has previously been identified as the optimal day for the identification of $IgE^+/B220^+$ cells (Manetz & Meade, 1999). Draining lymph nodes were collected (left and right nodes/animal) in 2 ml PBS and dissociated using frosted ends of two microscope slides. Cell counts were performed using a Coulter Counter (Z2 model, Beckman Coulter, Fullerton, CA), and 10^6 cells/sample were added to wells of a 96-well plate. Cells were washed in PBS and then incubated with Live/Dead Fixable Far Red Dead Cell Stain (Life Technologies, Carlsbad, CA) in PBS for exclusion of non-viable cells. Cells were washed using flow staining buffer (1% bovine serum albumin/0.1% sodium azide in PBS) and then incubated with F_c block (clone 2.4G2). The cells were then incubated on ice in flow staining buffer with a combination of the following antibody conjugates: anti-CD45R/B220 (V500 or Alexa Fluor 700, clone RA3-6B2, BD Biosciences, San Jose, CA), anti-CD3 (V500, clone 500A2, or APC, clone 145-2C11, BD), anti-CD4 (PE-Cy7, clone GK1.5, eBioscience, San Diego, CA), anti-CD8 (APC-H7 or PE, clone 53-6.7, BD), and anti-IgE (FITC, clone R35-72, BD). Cells were washed and fixed in cell fixation buffer (BD). For analysis, cells were re-suspended in staining buffer and analyzed on a BD Biosciences LSR II flow cytometer. All data were analyzed using FloJoe software. A minimum of 10 000 events per sample was collected.

Total serum IgE

Following euthanasia of animals used in the 10-day phenotypic analysis assay, blood samples were collected via cardiac puncture; sera were separated by centrifugation and frozen at –20 °C for subsequent analysis of serum IgE. A standard colorimetric sandwich ELISA was performed as previously described (Franko et al., 2012).

Statistical analysis

Data were first tested for homogeneity using the Barlett's ChiSquare test. If homogeneous, a one-way analysis of variance (ANOVA) was conducted. If the ANOVA showed significance at $p \leq 0.05$, the Dunnett's Multiple Range t-test was used to compare treatment groups with the control group. For dose response studies, linear trend analysis was performed to determine if uITO had exposure concentration-related effects for specific end-

points. Differences were considered to be significant if p+0.05 as compared to vehicle controls. A Student's t-test was used when comparisons were made between two groups.

Results

Toxicity study

Exposure of breached skin to 10% uITO in DMSO produced no changes in body weight, systemic signs of toxicity, or visual signs of inflammation at the exposure sites (data not shown).

Lymphocyte proliferation following dermal exposure to uITO

A dose-responsive increase (Linear Trend Test; p<0.01) in proliferation was observed in animals exposed to increasing concentrations of uITO through both intact and breached skin (Figure 1). Similar levels of lymphocyte proliferation – as represented by [^{3}H]-thymidine incorporation – were observed for the high dose groups (10% uITO) following exposure to intact and breached skin (2072 and 2036 dpm, respectively). However, the stimulation indices (SI) for these two exposures differed considerably with an SI of 5.7 following exposure to 10% uITO for the intact skin group and 1.8 for the breached skin group. The difference in SI following the two methods of dermal exposure can be explained by the increased proliferation observed following DMSO exposure to breached skin compared to intact skin (1134 vs 365 dpm, p<0.01). Using data from the intact exposure groups, the calculated EC3 value for uITO was 4.7%. The positive control group (HCA) had an SI of 27, within the historical range for our laboratory (data not shown).

Lymphocyte proliferation following intradermal exposure to ITO

Given the variability in response between studies following dermal exposure, additional studies were conducted following intradermal administration of the test article in PBS to eliminate the DMSO effect and to increase bioavailability. A significant response (p<0.01) resulting in an SI of 6.1 was seen following exposure to 5% uITO, with no further increase in proliferation observed in the 10% dose group (Figure 2). No signs of systemic toxicity including body weight changes were observed in any of the mice in the study. There was a visibly noted dose-responsive erythema at the injection site, with no erythema in mice exposed to 2.5% uITO, a majority of mice exposed to the 5% dose demonstrating mild erythema, and mice exposed to a 10% level having mild-to-moderate erythema. In all cases, the erythema resolved by the day of euthanasia. At euthanasia, intradermal deposits of test article were visible in all mice exposed to any concentration of uITO.

Phenotypic analysis of draining lymph node cells and total serum IgE levels following intradermal exposure to uITO

Phenotypic analysis of draining lymph node cells was undertaken to begin to understand the mechanism of immune activation following exposure to uITO. Based on the results of the LLNA intradermal exposure study, a concentration of 5% uITO was chosen for this study. Consistent with lymphocyte proliferation data measured by incorporation of [^{3}H]-thymidine, total cell numbers were significantly elevated (p<0.05) at the Day 6 timepoint (5.7 [±0.3] × 10^6 for uITO-exposed vs 2.4 [±0.1] × 10^6 for VH-exposed animals). By Day 10, total cell

numbers increased ($p<0.01$), with counts reaching 14.0 [±1.8] × 10^6 in uITO mice compared to 3.5 [±0.7] × 10^6 for the vehicle hosts. Consistent with results from well characterized T-cell-mediated sensitizers, at Day 10 an increase in absolute numbers (3.1 [±0.4] × 10^6 vs 0.5 [±0.1] × 10^6; $p<0.01$) and percentage (23.3 [±0.9]% vs 12.5 [±0.9]%; $p<0.01$) of $B220^+$ cells and a moderate increase in absolute numbers ($p<0.01$) with a decrease ($p<0.05$) in the percentage of both $CD4^+$ (6.4 [±0.8] × 10^6 {48.4 [±0.7]%} vs 1.9 [±0.4] × 10^6 {55.1 [±2.3]%}) and $CD8^+$ (3.3 [±0.5] × 10^6 {24.8% ± 0.6%) vs 1.1 [±0.3] × 10^6 {29.7 [±1.2]%}) T-cells were observed in mice exposed to 5% uITO compared to in vehicle control counterparts. No significant increases in $B220^+/IgE^+$ cell absolute numbers/percentages or total serum IgE levels was noted (Table 2).

Discussion

Given the recent recognition of lung pathology associated with exposure and the lack of understanding of the pathophysiology of the disease process, these studies were undertaken to begin to investigate the potential immunomodulatory effects of ITO. A dermal model based on the LLNA was chosen to initiate the investigations based on the large LLNA database available for chemicals inducing both T-helper (T_H)-1 and T_H2 cell-mediated responses following this route of exposure and concern for the role of dermal exposure in development of the sensitization phase of several T-cell-(e.g., chronic beryllium disease; Tinkle et al., 2003; Cummings et al., 2007; Thomas et al., 2013) and IgE-(i.e., based on responses to latex (allergy); Woolhiser et al., 2000; Howell et al., 2002), acid anhydrides (Zhang et al. 2004), or isocyanates (Petsonk et al., 2000) mediated pulmonary diseases. Additionally, the easily isolated lymph node draining the ear provides a source of cells for mechanistic studies (Manetz & Meade, 1999).

Based on information provided by the supplier (Sigma), the uITO used in these studies was ≤ 50 nm. Although reports on the potential for nanoparticles to penetrate the skin have been mixed (reviewed by Crosera et al., 2009), using a static in *vitro* diffusion system, Larese et al. (2009) demonstrated penetration of silver nanoparticles (ranging from 9.8–48.8 nm) through both intact and damaged full-thickness human skin. To simulate a more realistic workplace exposure paradigm, using an *in vitro* model with addition of mechanical flexion of the skin, Tinkle et al. (2003) demonstrated epidermal penetration of particles as large as 1.0 μm. Penetration into the epidermal layer allows for Langerhans cells (skin antigen-presenting cell) activation and immune activation. In these *in vivo* studies, using lymphocyte proliferation as a biomarker of exposure, data support a potential for uITO nanoparticles to penetrate both breached and intact skin.

Although animal studies investigating the potential for uITO to induce hypersensitivity responses have not previously been published, following pulmonary exposure to sintered ITO, toxicity studies have demonstrated hyperplasia of mediastinal lymph nodes with increased areas of lymphoid follicles and increased numbers of lymphocytes in mice (Nagano et al., 2011b) and the development of granulomas in bronchus-associated lymphoid tissue and mediastinal lymph nodes in rats (Nagano et al., 2011a,c). The cumulative results from the studies presented here support the potential for uITO to induce T-cell-mediated sensitization. Following dermal exposure to uITO, a dose-responsive increase in lymphocyte

proliferation was observed (reaching statistical significance and SI >3 at both 5% and 10% levels). The EC3 calculated for uITO was 4.7%, resulting in its classification as a mild sensitizer (Loveless et al., 2010). This classification is similar to other metals including nickel, which are known human sensitizers. Nickel chloride has been reported to have an EC3 of 3.5% (Basketter et al., 1994) and an EC3 of 2.5% has been reported for nickel sulfate (Ryan et al., 2002). Providing additional support for a T_H1-mediated mechanism, phenotypic analysis of draining lymph node cells was consistent with that observed following exposure to well characterized T-cellmediated sensitizers with an increase in the $\%B220^+$ cells and a decrease in the $\%CD4^+$ and $\%CD8^+$ cells and no significant elevation in $IgE^+/B220^+$ cells (Manetz & Meade, 1999). This was further supported by absence of an elevation in total serum IgE in the uITO-exposed mice.

In addition to pulmonary alveolar proteinosis and other lung pathology, analysis of clinical, radiological, and histopathological data from cases of workers with indium lung disease revealed an increased cellularity of broncho-alveolar lavage fluid with a predominance of lymphocytes and the presence of granulomas associated with cholesterol clefts (Cummings et al., 2012). Previous findings in animal studies combined with these data demonstrating the potential for uITO to induce a T-cell mediated response in mice, raise concern for the potential for immunologically-mediated disease following occupational exposure to ITO. These studies provide further support for the need to better understand the natural history and pathophysiology of ITO mediated pulmonary disease.

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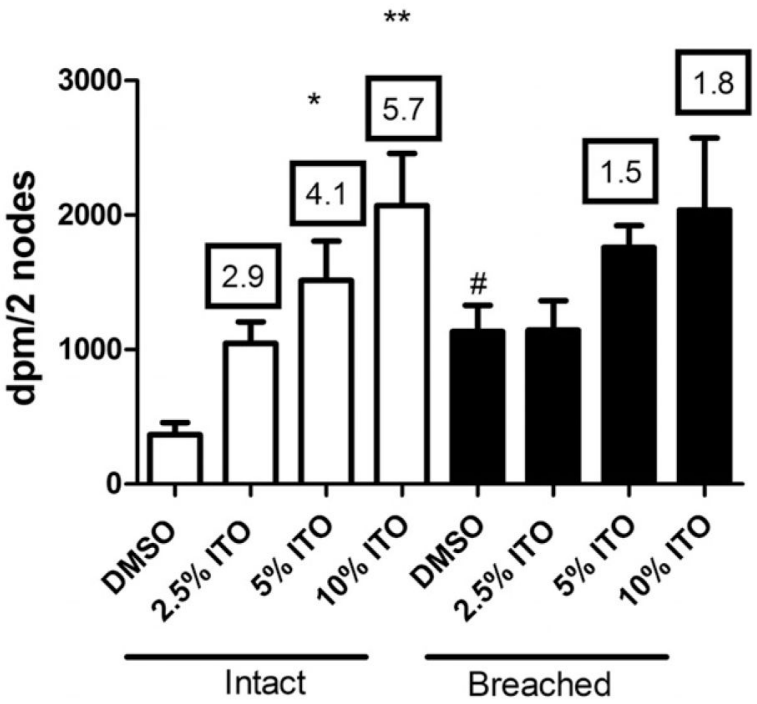

Figure 1.

Lymphocyte proliferation. Proliferation was assessed by [^{3}H]-thymidine incorporation into draining lymph node cells following dermal intact or breached skin exposure to vehicle (DMSO) or uITO. Bars represent means ± SE of five mice/group. Numbers appearing above the bars are the stimulation indices for respective concentration tested. Levels of statistical significance are denoted by *p<0.05 and **p<0.01 as compared to corresponding vehicle; #designates significant difference between animals exposed to DMSO or the same concentration of test article through breached vs intact skin. An EC3 of 4.7% was calculated for exposure to intact skin.

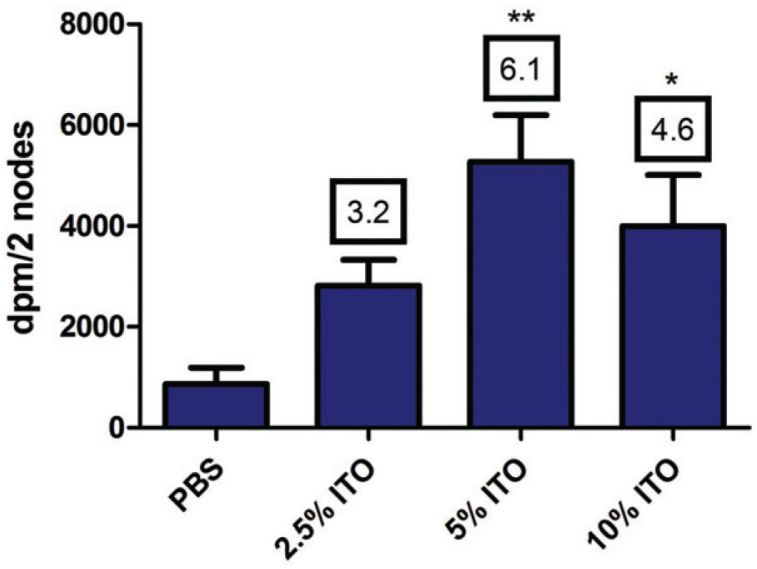

Figure 2.

Lymphocyte proliferation. Proliferation was assessed by [^{3}H]-thymidine incorporation into draining lymph node cells following intradermal exposure to vehicle (PBS) or uITO. Bars represent means ± SE of five mice/group. Numbers appearing above the bars are the stimulation indices for respective concentration tested. Levels of statistical significance are denoted by *p<0.05 and **p<0.01 as compared to vehicle control.

Table 1

Examples of known hypersensitivity responses to metals.

Metal	Hypersensitivity response	Associated disease
Platinum[a]	Type I, Type IV	Urticaria, Asthma
Nickel[b]	Type IV	Allergic contact dermatitis, Asthma
Cobalt[c]	Type IV, Type I	Allergic contact dermatitis, Asthma
Beryllium[c]	Type IV, Type I	Chronic beryllium disease
Chromium[c]	Type IV	Allergic contact dermatitis
Gold[c]	Types II, III, IV	Allergic contact dermatitis, 'burning mouth syndrome' in those with gold dental restorations, autoimmune thrombocytopenia
Palladium[c]	Type IV	Allergic contact dermatitis
Mercury[c]	Type IV	Allergic contact dermatitis

[a] Makrilia et al. (2010);

[b] Büdinger and Hertl (2000);

[c] Nordberg et al. (2011).

Table 2

Phenotypic and total serum IgE analysis.

	Day 6		Day 10	
End-point	**Vehicle**	**5% uITO**	**Vehicle**	**5% uITO**
Cell counts				
Total ($\times 10^6$)	2.4 ± 0.1	5.7 ± 0.3*	3.5 ± 0.7	14.0 ± 1.8**
$B220^+$ cells absolute ($\times 10^6$) (%)	0.3 ± 0.0 12.4 ± 1.0	1.1 ± 0.1* 19.0 ± 1.2*	0.5 ± 0.1 12.5 ± 0.9	3.1 ± 0.4** 23.3 ± 0.9**
IgE^+B220^+ cells, %	Not Tested	Not Tested	4.6 ± 2.3	7.1 ±5.1
$CD4^+$ cells absolute ($\times 10^6$) (%)	1.4 ± 0.07 58.7 ± 0.6	2.9 ± 0.1* 50.4 ± 1.0*	1.9 ± 0.4 55.1 ± 2.3	6.4 ±0.8** 48.4 ±0.7*
$CD8^+$ cells absolute ($\times 10^6$) (%)	6.9 ± 0.4 29.1 ± 0.4	1.7 ± 0.0* 29.6 ± 0.3	1.1 ± 0.3 29.7 ± 1.2	3.3 ± 0.5** 24.8 ± 0.6*
Total serum IgE (ng/ml)	Not Tested	Not Tested	329 ± 94	394 ± 85

Values represent the mean ± SE from five animals per group. Statistical significance is designated as

* $p \leq 0.05$

** $p \leq 0.01$.