

Altered Zinc Homeostasis and Caspase-3 Activity in Murine Allergic Airway Inflammation

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Zn may have an important protective role in the respiratory epithelium and Zn deficiency may enhance airway inflammation and epithelial damage. The effects of mild nutritional Zn deficiency on airway hyperresponsiveness (AHR) and airway inflammation in mice sensitized and challenged with ovalbumin (OVA) to induce an allergic response were investigated. Balb/c mice were given Zn normal (ZN, 50 mg/kg Zn) or Zn limited diets (ZL, 14 mg/kg Zn) before and during induction of allergic airway inflammation, with appropriate controls (saline-treated, SAL). ZL mice had greater levels of AHR than ZN mice, regardless of presence or absence of allergic inflammation. These mice also had increased eosinophilia and mucus cell hyperplasia compared with ZN mice. Second, ZN and ZL OVA-treated mice had significant decreases in airway epithelial Zinquin fluorescence, indicating a lowered availability of Zn compared with their SAL-treated counterparts. In contrast, the pro-apoptotic protein caspase-3, which was co-localized with Zn in the apical epithelium, was significantly increased in both ZN and ZL OVA-treated mice. Immunologically active caspase-3 and apoptosis were increased in OVA-treated mice, especially the ZL group. These findings provide the first data for adverse effects of Zn deficiency on the respiratory epithelium and support a role for altered Zn homeostasis and caspase upregulation in asthma.

Asthma is a chronic inflammatory disorder of the airways in which the recruitment and activation of inflammatory cells such as eosinophils, mast cells, and neutrophils play a major role in the pathogenesis of this disease (1). Suboptimally-controlled asthma is also accompanied by hypersecretion of mucus, variable airways obstruction, and enhanced airway hyperresponsiveness (AHR). One major pathologic feature of asthma is the shedding and desquamation of the respiratory epithelium. Bronchial biopsies taken from patients with asthma commonly demonstrate loss of the mucosal epithelium, and the swelling of ciliated columnar cells, giving the epithelium a "fragile appearance" (2). It has been suggested that increased release of

serine proteases from neutrophils recruited during airway inflammation may be responsible for the detachment of human epithelial cells from their basement membrane (3). Because apoptotic pathways can be targeted by specific inhibitors (4, 5), it is important to determine whether epithelial cells die by apoptosis resulting in shedding. Although it is a normal process, the regulation of apoptosis becomes altered in some chronic diseases, such as HIV and diabetes (6). Recently, it has been shown that the apoptotic caspase-3 protein was increased in the epithelium and submucosa of human bronchial biopsies from individuals with asthma, both steroid-treated and -untreated, when compared with subjects without asthma (7).

Zn is an important regulator of caspase-3, as well as an antioxidant, microtubule stabilizer, growth co-factor, and anti-inflammatory agent (8). Over the past 30 yr, many researchers have demonstrated the important role of Zn in a variety of physiologic processes, including growth and development, maintenance and priming of the immune system, and in tissue repair and regeneration. Zn is one of the most widely distributed biometals found in living tissues and secretions and is transported loosely bound to albumin in the circulation (9). In our previous studies, using a novel Zn-specific fluorophore (Zinquin), we have shown that Zn is a cellular regulator of caspase-3 activation and that it is co-localized with the zymogen form of caspase-3 in the apical cytoplasm of sheep and human airway epithelial cells (10). Furthermore, depletion of available Zn in these cells leads to rapid activation of caspase-3, culminating in apoptosis (11).

The increase in prevalence of asthma has been linked to environmental factors, including diet (12). Several studies have reported an association between asthma and low hair and serum Zn levels (13–17). In another study, a negative relationship was found between wheezing and serum Zn:Cu ratio in a large population within the United States ($n = 9,074$) (18). Furthermore, in an investigation into the relationship between allergic diseases and dietary antioxidants, it was noted that there was an increase in the presence of atopy, bronchial reactivity, and the risk of allergic-type symptoms in adults with the lowest intake of dietary Zn (19). The significance of the association between asthma symptoms, low serum Zn levels, and low dietary Zn intake is not yet fully understood.

In this investigation, we have used a well-established murine model of allergic airway inflammation (20) to examine: (i) the influence of dietary Zn on AHR and airway inflammation in saline (SAL)-treated and ovalbumin (OVA)-treated mice, and (ii) the influence of allergic in-

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Abbreviations: airway hyperreactivity, AHR; bronchoalveolar lavage fluid, BALF; basement membrane, BM; bovine serum albumin, BSA; fluorescein isothiocyanate, FITC; grey scale intensity units, GSU; Hanks' balanced saline solution, HBSS; high power field, HPF; lumen, LM; metallothionein, MT; Optimum Cooling Tissue Medium, OCT; ovalbumin, OVA; phosphate-buffered saline, PBS; saline, SAL; *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethyl-enediamine, TPEN; Zn-limited, ZL; Zn normal, ZN; zinc, Zn.

flammation *per se* on Zn and pro-caspase-3 levels in respiratory epithelial cells.

Materials and Methods

Animals

Specific pathogen-free 4-wk-old female Balb/c mice were purchased from the University of Adelaide Animal Center, Adelaide, South Australia. Weight-matched mice were housed together in groups of eight in plastic cages with stainless steel grid floors (38 cm × 25 cm, 5 mm mesh, 1 mm wire diameter to minimize coprophagy). Animals were housed at 21°C with a 14-h light/10-h dark cycle. All experiments conformed to National Health and Medical Research Council Guidelines and were approved by the animal ethics committees of The Queen Elizabeth Hospital and The University of Adelaide.

Induction of Mild Zn Deprivation and Allergic Airway Inflammation in Balb/c Mice

Induction of Zn deprivation was performed using the method of Coyle and coworkers (21) replacing egg white with casein protein (Bonlac Foods, Victoria, Australia). Mice were acclimatized for 7 d and given a 20% casein diet containing 50 mg of Zn per kg of diet (Table 1). To prevent exogenous Zn contamination, all grids, mice cages, and water bottles were prewashed with milliQ water. Mice were then either fed this ZN diet or given a zinc-limited (ZL) diet containing 14 mg of Zn per kg diet for the duration of the experiment.

Allergic inflammation was induced using the method of Xiong and coworkers (20) with modifications (Figure 1). Briefly, mice on both ZN and ZL diets were injected intraperitoneally on Days 1 and 12 with 50 µg of OVA (Sigma-Aldrich, Sydney, Australia) per 1 ml of alhydrogel (CSL, Parkville, Australia) in 0.9% sterile saline. SAL-treated mice received alhydrogel in 0.9% sterile saline alone. Sensitized mice (OVA-treated) were then aerochallenged with 10 mg/ml of OVA in 0.9% saline from Day 19 to Day

TABLE 1
Composition of diets

Ingredient	Zn Normal (50 mg/kg Zn)	Zn Limited (14 mg/kg Zn)
	g/kg	
Casein*	200	200
Cellulose	50	50
Corn starch	430	430
Choline Bitartrate	2.5	2.5
L-Cysteine	3	3
Soybean Oil†	70	70
Sucrose	200	200
AIN 93M Mineral Mix‡	35	35
AIN 93VX Vitamin Mix§	10	10
ZnCO ₃	0.062	0

* The Zn content of the casein diet before addition of ZnCO₃ was 14 mg/kg as determined by atomic absorption spectroscopy (AAS). This is due to the Zn content of the casein used. The Zn content of the Zn normal diet was 50 mg/kg as determined by AAS.

† Lion and Globe, Hop Hing Oil Factory, Kowloon, Hong Kong.

‡ AIN 93M Mineral mix profile (g/kg diet): KH₂PO₄, 17.155; CaCO₃ 14.645; NaCl, 12.530; MgSO₄ · 7 H₂O, 4.99; FeC₂H₃O₇ · 5 H₂O, 0.296; CaPO₄, 0.170; MnSO₄ · 4 H₂O, 0.080; CuSO₄ · 5 H₂O, 0.123; KI, 0.00025; (NH₄)₆Mo₇O₂₄ · 4 H₂O, 0.00125; Na₂SeO₃, 0.0005.

§ AIN 93VX vitamin mix profile (mg/kg diet): thiamine-HCl, 70; riboflavin, 30; nicotinic acid, 50; pantheric acid, 150; pyridoxal HCl, 15; hydroxocobalamin, 0.02; inositol, 400; P-aminobenzoic acid, 50; folic acid, 10; biotin, 0.4; cholecalciferol, 0.01; glucose, 225; and retinol palmitate, 2900 U/kg (ICN Biochemicals Australasia, Seven Hills, NSW, Australia).

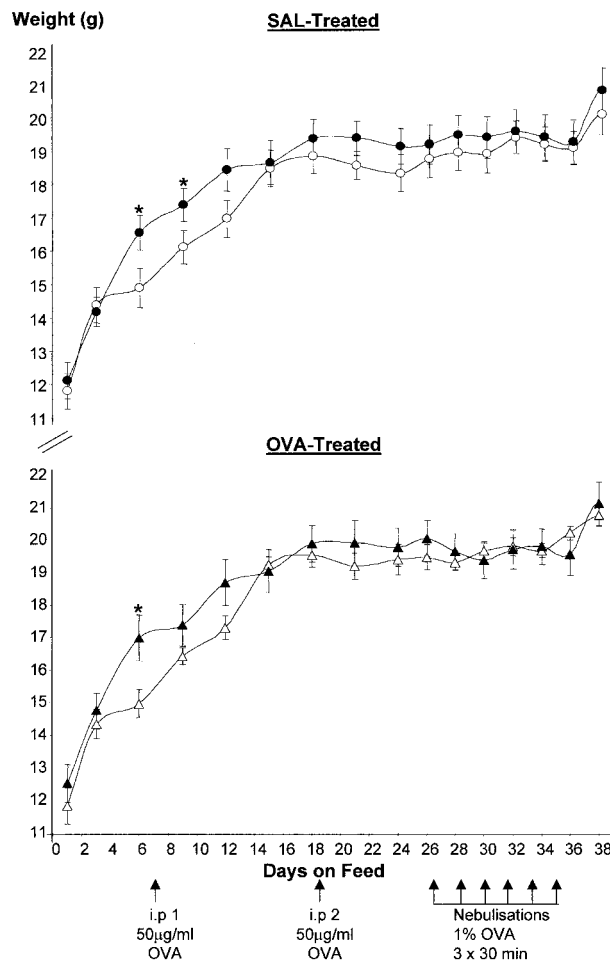


Figure 1. Effect of Zn diets on weight patterns. Figure shows the slower weight gain of ZL mice in the first 2 wk on the diets. The x axis indicates days on the diets and the y axis the corresponding body weights in grams (g). Filled symbols indicate ZN mice and open symbols ZL mice (n = 8). Upper panel represents data for the SAL-treated mice and lower panel that for the OVA-treated mice. The protocol for sensitization and nebulization is shown in the lower panel. Results represent the means ± SEM for eight mice per group, Student's *t* test, **P* ≤ 0.05.

36, three times a day for 30 min every second day using a side-stream nebulizer, which produced particles of 1–3 µm (Fisher and Paykel, Sydney, Australia). Mice were tested for AHR on Day 37, bled by cardiac puncture and killed by cervical dislocation for collection of tissues. Eight mice were used per group.

Assessment of AHR to Methacholine by a Barometric Whole Body Plethysmograph

AHR to the bronchoconstrictor β-methacholine was assessed in conscious, unrestrained mice by barometric plethysmography using equipment and software from Buxco (Troy, NY). This system yields a dimensionless parameter known as enhanced pause (Penh) that reflects changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration. In OVA-treated mice, Penh is correlated with increased eosinophilia and other inflammatory indices and is used to empirically monitor airway function as described previously (22, 23). Briefly, mice were placed in a

chamber and exposed to an aerosol of water (baseline readings) and then cumulative doubling concentrations of methacholine (dissolved in water to make concentrations in solution ranging from 3.15–50 mg/ml). The aerosol was generated by an ultrasonic nebulizer and drawn through the chamber for 2 min at a constant flow rate. The inlet was then closed and Penh readings were taken for 3 min and averaged.

Assessment of Bronchoalveolar Lavage Fluid Inflammatory Cell Infiltration

After anesthesia, the trachea was cannulated with a blunted 18.5-gauge needle and lavage performed by perfusing the lungs twice with 1 ml of $1\times$ Hanks' buffered saline solution (HBSS, pH 7.4) and fluid withdrawn into tubes and stored on ice. The lavage fluid was centrifuged at 1,500 rpm for 5 min at 4°C and the cell pellet resuspended with 500 μ l of $1\times$ HBSS, total cell count measured, and cytospin preparations were performed. Cells were stained with May Grünwald (Sigma-Aldrich) and Giemsa (Sigma-Aldrich), and a differential count of 200 cells was performed in duplicates using standard morphologic criteria.

Assessment of Histology of Lung Tissue

Tissues for histopathologic assessment were collected 24 h after the final aerosolization. Blood from the pulmonary capillary bed of killed mice was removed by injecting 2 ml of 0.9% saline directly into the apex of the heart, across the interventricular septum and into the right ventricle. Lungs were then inflated using a blunted 18.5-gauge needle with 1 ml of 10% phosphate-buffered formalin (Sigma-Aldrich) for paraffin sections or with Optimum Cooling Tissue Medium (OCT; Tissue-Tek, Tokyo, Japan) for cryostat sections and processed accordingly.

After an overnight fixation with formalin, lung tissues representing the central and the peripheral lung tissue were horizontally sliced from the midzone of a single lobed lung and embedded in paraffin. Ribbons of 5 μ m thickness were cut and stained with Charbol's chromotrophe hematoxylin (Sigma-Aldrich) for identification of eosinophils and Alcian blue/periodic acid-Schiff (Sigma-Aldrich) for enumeration of mucin-secreting cells. Eosinophils and mucus-containing cells were identified by morphologic criteria as described by Foster and colleagues (23).

For frozen tissues, the entire lung was excised and sections of the trachea and lobes quickly placed into plastic embedding trays containing OCT and snapped frozen in a glass beaker containing 50 ml of isopentane (BDH-Merck, Victoria, Australia) by immersion into liquid nitrogen. Frozen tissue blocks were sectioned at 8 μ m thickness and allowed to adhere onto poly-L-lysine (Sigma-Aldrich)-coated glass slides at room temperature for 20 min. Sections were fixed in 100%-chilled acetone (Sigma-Aldrich) for 10 min at room temperature before tissue staining.

Detection of the Pro-caspase-3 and Active Caspase-3 Protein Levels by Immunohistochemistry

Caspase-3 protein expression was determined using the primary rabbit anti-human precursor 32 kD caspase-3 antibody (Pharmin-gen, Becton-Dickinson, San Diego, CA) and a primary rabbit anti-human active 17 kD caspase-3 antibody (New England BioLabs, Beverly, MA) both of which were cross-reactive with mice. Cryostat lung sections were washed three times for 5 min each with a 2% solution of bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.4) and incubated with a 1:250 dilution of the primary antibody overnight at 4°C. Visualization was performed using a 1:100 dilution of a goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Pharmin-gen, Becton-Dickinson) for 1 h at room temperature in a humidified atmosphere. Negative controls lacked the primary antibody.

Detection of a Caspase Cleavage Product of Cytokeratin 18 by Immunohistochemistry

To confirm apoptosis, the M30 Cytodeath Kit (Roche Diagnostics, Mannheim, Germany) was used on acetone-fixed cryostat sections of mice lung tissue. This kit uses a mouse IgG_{2b} monoclonal antibody which detects an epitope formed during the cleavage of cytokeratin 18 (CK18) by caspases in the early stages of apoptosis. The primary antibody was used at a final dilution of 1:50 incubated for 18 h at 4°C; an anti-mouse FITC-conjugated secondary antibody (gift of Dr. C. Murgia, Institute for Food and Nutrition, Rome) was used at a dilution of 1:150 for 1 h at 4°C in a humidified atmosphere. Negative controls lacked the primary antibody.

Detection of Intracellular Zn Levels Using the Zn Fluorophore Zinquin

Cryostat sections were labeled with ethyl-(2-methyl-8-p-toluene-sulfonamido-6-quin-olyloxy) acetate (Zinquin; Department of Chemistry, University of Adelaide, Adelaide, Australia), as previously described (11). Briefly, Zinquin was freshly diluted in PBS to a final concentration of 25 μ M and immediately pipetted onto these sections. After incubation for 30 min at room temperature in dark and humidified conditions, sections were washed three times with milliQ water and mounted with an anti-fade fluorescence mounting medium (DAKO Corporation, Carpinteria, CA). An autofluorescence control (PBS alone) was set up for each section as a negative control.

We have called the component of cellular Zn accessible to Zinquin binding "available Zn." Previous experiments have shown that Zinquin fluorescence is completely quenched by the addition of the Zn chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethyl-enediamine (TPEN). Available Zn is unlikely to include the major pool of metallothionein Zn because it has been shown in liver cytosols that Zinquin fluorescence is greatly quenched by metallothionein (24).

Co-localization of Pro-caspase-3 and Zn

In some instances, dual immunohistochemistry was performed. Briefly, after the pro-caspase-3 immunostaining procedure was completed, sections were washed three times in PBS to remove excess secondary antibody and incubated sequentially with 25 μ M Zinquin for 30 min at room temperature in dark and humidified conditions. Sections were then re-washed in milliQ water and permanently mounted with fluorescent mounting medium.

Image Analysis

All slides were examined using standard epifluorescence microscopy and UV laser confocal microscopy. A Bio-Rad MRC-1000 UV Laser Scanning Confocal Microscope System, equipped with a UV-Argon laser, was used in combination with a Nikon Diaphot 300 inverted microscope in fluorescence mode. For Zinquin, fluorescence excitation was at 351/8 nm and emission at 460 LP, whereas for FITC fluorescence was at excitation 488/10 nm and emission at 522/35. Images were collected using a 40 \times water immersion objective lens with NA 1.15. Each image was averaged over six scans by Kalman filtering. Before the capturing of images, the laser settings of the confocal microscope were adjusted to a level which excluded background fluorescence. The laser strength for Zinquin fluorescence was 3%, and for FITC fluorescence the laser was 10%. Where dual staining was performed, fluorescence images were merged to demonstrate co-localization of Zinquin and pro-caspase-3 staining using Confocal Assistant (Version 4.02; Leading Edge Pty Ltd., Marion, South Australia) software package. For each image, five randomly positioned profile lines were drawn at 90° to the epithelial surface so that they spanned the entire epithelium. The mean fluorescence intensity

was calculated for each of 15 evenly spaced intervals across the epithelium, the first beginning at the luminal surface and the last terminating at the basement membrane. Apical region is defined as intervals from 1 to 5 and the basal region as intervals from 6 to 15. Fluorescence was quantified using the CoMOS software package (Bio-Rad, Hemel Hempstead, UK) and results expressed as gray scale intensity units (GSU) between 0 and 255 units. The mean fluorescence intensity was collated from at least two mice per group, each having five to six airways assessed.

Determination of Serum and Liver Zn Levels by Atomic Absorption Spectroscopy

Serum and liver Zn levels were determined as previously described (25). Serum Zn levels were determined using a Graphite Atomic Absorption Spectroscopy (Perkin-Elmer, Uberlinger, Germany). Liver samples were diluted with 5 vols of cold Tris HCl buffer (0.01M, pH 8.2) and homogenized using a Potter-Elvehjem homogeniser (Wheaton, NJ). Aliquots of liver homogenate were then dried at 70°C for 48 h before nitric acid digestion as appropriate for Zn analysis by flame atomic absorption spectroscopy (303 Atomic Absorption Spectrophotometer; Perkin-Elmer).

Statistical Analysis

Data are expressed as means ± SEM. When suitable, results were interpreted using ANOVA with Tukey's *post hoc* test using the General Linear Model on Minitab (Minitab, State College, PA). Where appropriate, the Student's *t* test was performed. Differences were considered statistically significant at *P* ≤ 0.05, unless otherwise stated.

Results

The main body of results refer to four groups of mice on a protocol of dietary Zn manipulation and allergic sensitization and challenge (Figure 1B). For each parameter, two major comparisons were made:- (i) between SAL-treated and OVA-treated mice, and (ii) between diets (ZN versus ZL).

Body Weight

There was no effect of allergy on the weights of the ZN mice during the duration of the experiment. The curves for OVA-treated versus SAL-treated groups were similar, indicating that the sensitization and nebulization protocol has not affected growth (Figure 1).

There was a separation of weights (*P* ≤ 0.05) between ZL mice (*open symbols*) and ZN mice (*closed symbols*) during the first 2 wk of dietary exposure (Figure 1), consistent with known effects of Zn deficiency on growth. This separation was found for both SAL-treated (*upper panel*) and OVA-treated (*lower panel*) mice and occurred during the phase of maximal growth and sensitizations to OVA, but before nebulizations. At later time points, body weights plateaued and Zn deprivation had no significant effect on them.

Eosinophilia

A major criterion of allergic inflammation, eosinophilia, was examined by performing differential counts on leukocytes in BALF and peripheral blood, and by counting eosinophils around airways.

The ZN OVA-treated mice had marked, highly significant increases in eosinophils in BALF, blood, and lung tissue, compared with SAL-treated mice (Table 2), confirming the success of the allergy induction protocol. BALF cells in ZN OVA-treated animals comprised a mean ± SEM of 73.2 ± 1.9% eosinophils, 17.4 ± 2.1% lymphocytes, 8.3 ±

TABLE 2
*Inflammatory parameters in SAL-treated and OVA-treated mice on different Zn diets**

	n	SAL-Treated		OVA-Treated [†]		RMSE [§]	ANOVA [‡] (<i>P</i> Value)		
		ZN ⁴	ZL	ZN	ZL		Allergy	Diet	Allergy × Diet
BALF	3	Percentage of Cells							
Eosinophils		ND	ND	73.2	73.3				
Neutrophils		ND	ND	1.2	0.6				
Lymphocytes		ND	ND	17.4	19.1				
Macrophages		ND	ND	8.3	7.0 [¶]				
Peripheral Blood	3	Percentage of Cells							
Eosinophils		1.3	1.5	5**	9.3 ^{¶,**}	0.9	0.000	0.012	0.019
Neutrophils		19.5	22.0	17.0	14.3**	2.8	0.042	0.950	0.224
Lymphocytes		78.8	75.0	77.8	75.3	2.5	0.852	0.121	0.745
Monocytes		0.3	1.0	0.5	1.3**	0.6	0.031	0.430	1.000
Lung Tissue	3	Cells/HPF							
Eosinophils		0.1	1.83 [¶]	41.4**	58.7 ^{¶,**}	4.6	0.000	0.007	0.019
Mucus-secreting cells		0.1	1.11 [¶]	70.6**	132.3 ^{¶,**}	5.8	0.000	0.000	0.000

Definition of abbreviations: SAL; saline, OVA; ovalbumin, ZN; normal Zn diet, ZL; Zn Limiting diet, BALF; bronchoalveolar lavage fluid, HPF; high power field. * Values represent means, *n* = 3 mice (duplicates).

[†] Mice were injected intraperitoneally with 50 µg of OVA on Days 1 and 12 followed by nine nebulizations with 1% OVA.

[‡] Two-way ANOVA using the factors Allergy (SAL-treated and OVA-treated), Diet (ZN, ZL).

[§] RMSE, root mean square error, is an estimate of the overall standard deviation. It is the square root of the mean square error term from the ANOVA calculations.

^{||} ND, not determined. For the BALF percentages in the non-allergic mice, too few cells were present and therefore an accurate percentage could not be determined.

[¶] Significantly different from ZN mice of the same allergy group.

** Significantly different from SAL-treated mice of the same diet group.

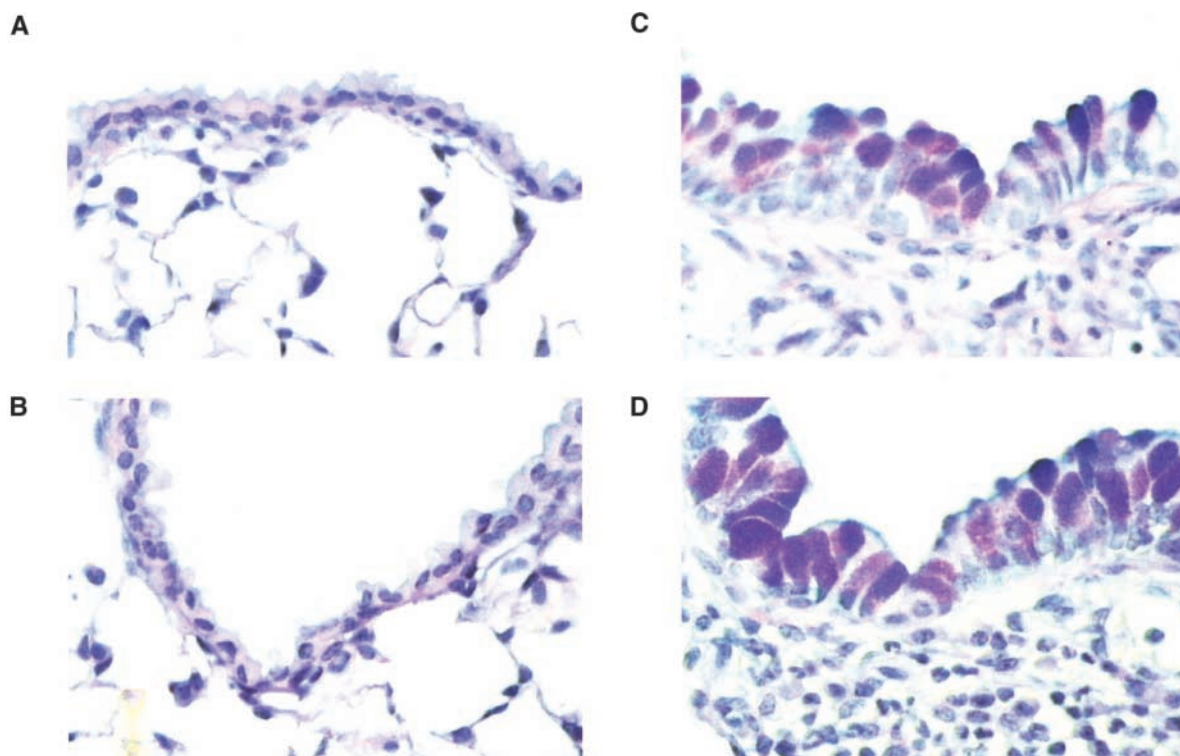


Figure 2. Effects of allergy and Zn status on mucus-secreting cell numbers. Figure shows the increase in mucus cells (stained purple with Alcian blue/periodic acid-Schiff) in the airways of the ZN OVA-treated (C), the ZL SAL-treated (B), and the ZL OVA-treated (D) compared with the ZN SAL-treated tissue (A). Original magnification: $\times 1,000$.

1.0% macrophages, and $1.2 \pm 0.6\%$ neutrophils; the number of cells recovered in the BALF fluid of ZN SAL-treated mice was too low to obtain accurate percentages (Table 2). There was no difference in the percentage of BALF eosinophils when SAL-treated or OVA-treated mice were placed on ZL diets. Thus, ZN OVA-treated mice had $73.2 \pm 1.9\%$ eosinophils compared with the ZL OVA-treated mice, which had $73.3 \pm 3.1\%$ (Table 2). Hence, dietary Zn did not influence BALF eosinophil percentages in these mice. There was no significant change in neutrophils, lymphocytes, or macrophages in the ZL OVA-treated mice.

In peripheral blood of ZN OVA-treated mice, eosinophils constituted $5 \pm 0.6\%$ of the cells, compared with $1.3 \pm 0.5\%$ in ZN SAL-treated mice ($P < 0.005$). There were no significant differences in percentages of lymphocytes, mono-

cytes, or neutrophils between ZN OVA-treated and ZN SAL-treated animals (Table 2). Tissue eosinophil counts were assessed in at least six different airways of three different mice using a $100\times$ objective lens and are expressed as mean number of eosinophils/HPF. ZN OVA-treated mice had a more than 400-fold increase in tissue eosinophils increasing from 0.1 ± 0.1 to 41.4 ± 4.4 ($P < 0.005$).

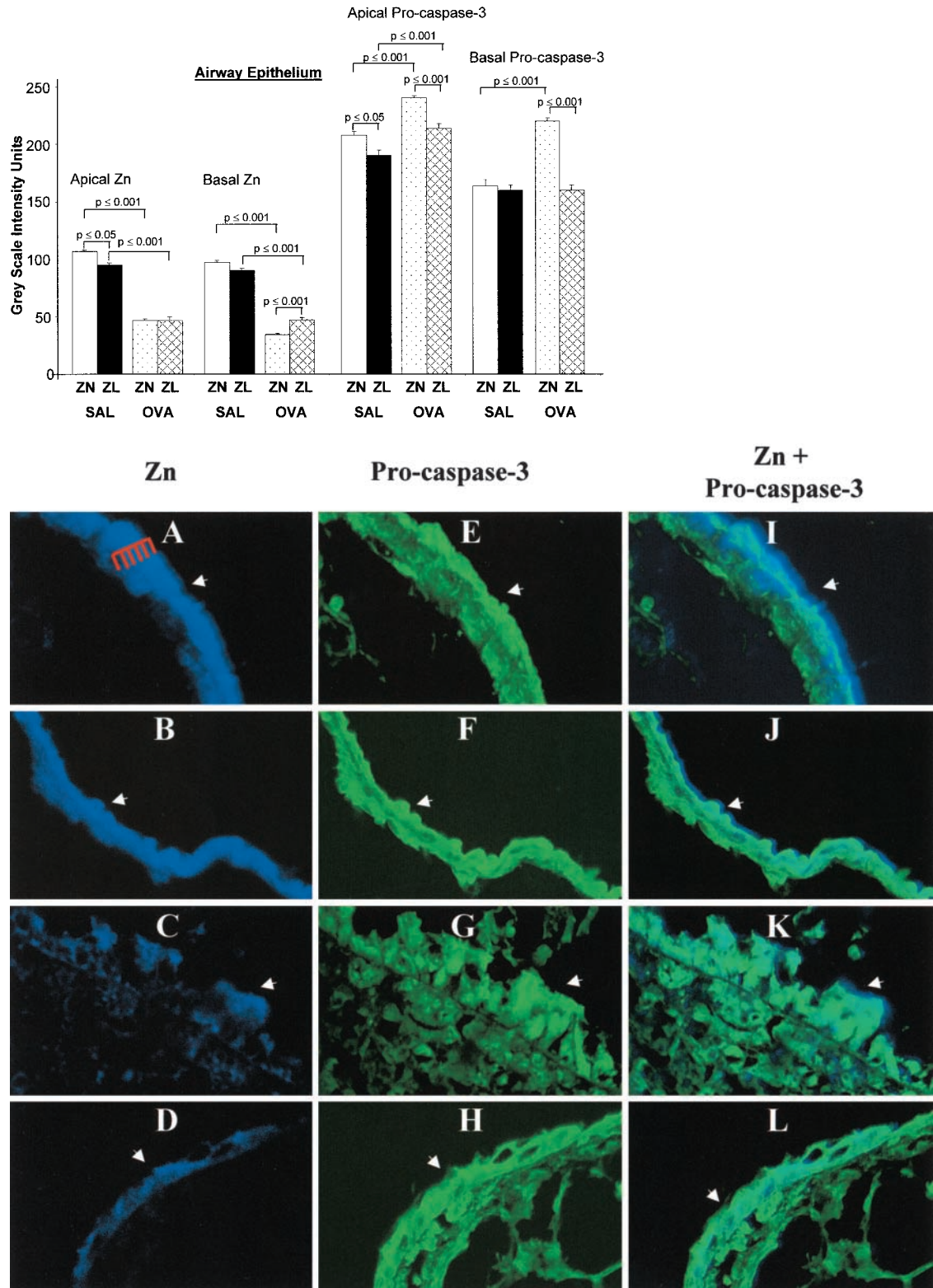
In contrast to BALF findings, Zn deprivation significantly increased blood eosinophil percentages in OVA-treated mice from $5.0 \pm 0.6\%$ in the ZN OVA-treated mice to $9.3 \pm 0.5\%$ in the ZL OVA-treated mice ($P \leq 0.005$, Table 2). In the SAL-treated mice, there were no significant differences in eosinophil percentages between mice on different Zn diets (Table 2). There were no significant differences for blood neutrophils and lymphocytes;

Figure 3. Effects of allergy and Zn status on available airway epithelial Zn and pro-caspase-3 levels. A shows the mean fluorescence intensities for apical and basal compartments of the epithelium for available Zn and pro-caspase-3 (for details see Materials and Methods). Note that the majority of the changes in available Zn and pro-caspase-3 affect both the apical and basal compartments. Histograms represent the means \pm SEM, $n = 8$. Note the general decreases in pro-caspase-3 levels of ZL OVA-treated mice compared with their ZN counterparts. Significant differences between groups are denoted by the P values stated. B shows a pronounced decrease in available Zn levels in airway epithelium of ZN OVA-treated (C) and ZL OVA-treated (D) compared with the SAL-treated counterparts (A and B, respectively). A decrease in the available Zn was found in the epithelium of ZL SAL-treated (B) when compared with ZN SAL-treated mice (A). However, there was no further decrease in available Zn levels in mice rendered both Zn limited and OVA-treated (D), compared with just OVA-treated alone (C). Conversely, there was a significant increase in pro-caspase-3 levels in airway epithelium of ZN OVA-treated (G) compared with the ZN SAL-treated (E). ZL OVA-treated mice (H) also had an increase in pro-caspase-3 protein levels compared with ZL SAL-treated mice (F). Confocal images were taken with a $\times 40$ objective lens and were labeled with Zinquin (A, B, C, and D) or with primary anti-pro-caspase protein detected with a secondary FITC-conjugated antibody (E, F, G, and H). I, J, K, and L shows the co-localization of available Zn (blue) and pro-caspase-3 (green) in a dual-labeled section of airway epithelium of mice. Typical images are shown; arrows indicate the luminal epithelium. The image size is $144 \times 96 \mu\text{m}$.

however, monocyte percentages were increased in both ZL SAL-treated and OVA-treated mice.

Consistent with an increase in blood eosinophils, there was a significant rise in the number of infiltrating tissue eosinophils around the airways of both SAL-treated and

OVA-treated mice given the ZL diet. First, in the SAL-treated groups, ZL mice had an increase in eosinophil numbers from 0.1 ± 0.1 cells/high power field (HPF) in ZN mice to 1.8 ± 0.6 cells/HPF ($P \leq 0.05$). In addition, Zn deprivation further increased airway tissue eosinophil



numbers in OVA-treated mice ($P \leq 0.05$). Thus, ZL OVA-treated mice had 58.7 ± 4.4 cells/HPF compared with 41.4 ± 4.9 cells/HPF in the ZN OVA-treated mice.

Mucus Cells

Mucus-secreting cells were counted in at least six airways of three mice from both SAL-treated and OVA-treated groups and are expressed as mucus cells/HPF.

Mucus cell numbers were significantly increased in OVA-treated mice (Table 2) with ZN OVA-treated mice having a mean mucus cell count of 70.6 ± 3.2 cells/HPF, compared with 0.1 ± 0.1 cells/HPF in ZN SAL-treated mice ($P \leq 0.001$).

Zn deprivation increased mucus cell numbers in both SAL-treated and OVA-treated groups (Table 2). There were increases in mucus cell numbers from 0.1 ± 0.1 cells/HPF in ZN SAL-treated mice to 1.1 ± 0.3 cells/HPF in ZL SAL-treated mice ($P \leq 0.05$) and 70.6 ± 3.2 cells/HPF in ZN OVA-treated mice to 132.3 ± 3.5 cells/HPF in ZL OVA-treated mice ($P \leq 0.005$). Figure 2 shows typical images of positively stained mucus-secreting cells counted in paraffin sections of ZN OVA-treated mice (C) and ZL OVA-treated mice (D). Note the relative paucity of mucus cells in ZN SAL-treated mice (A) and ZL SAL-treated mice (B).

Serum and Liver Zn

Serum Zn in ZN SAL-treated mice was 17.6 ± 5.1 μ M compared with 13.2 ± 3.4 μ M in the ZL SAL-treated mice, 13.5 ± 1.5 μ M in the ZN OVA-treated mice, and 15.8 ± 1.7 μ M in the ZL OVA-treated mice. The mean liver Zn levels were within the range of 445–488 nmol/g wet weight. For all comparisons, the small changes in Zn concentrations were nonsignificant. The initial growth curves of mice on the ZL diet, however, clearly indicate that the Zn contents of the diet limited growth rates during the period of maximum growth. This functional measure is more sensitive than the measurement of total tissue or serum Zn levels in adult mice.

Intraepithelial Available Zn

There was a decrease in Zinquin fluorescence from an average of 100.9 ± 3.2 GSU in the ZN SAL-treated mice, to 38.9 ± 1.9 GSU in the ZN OVA-treated mice ($P \leq 0.001$). This occurred in both the apical and basal compartments (Figure 3A).

Zn deprivation also significantly decreased Zinquin fluorescence, but the magnitude of the decrease was much less than that due to allergy alone. Zinquin fluorescence fell from 100.9 ± 3.2 GSU in the ZN SAL-treated mice to 92.05 ± 3.3 GSU in the ZL SAL-treated mice ($P \leq 0.05$). As with effects of allergy alone, decreases occurred in both the apical and basal compartments (Figure 3A). In the OVA-treated group, Zn deprivation did not further decrease Zinquin fluorescence (47.6 ± 0.8 GSU).

Figure 3B shows typical images of Zinquin fluorescence in airway epithelium of ZN SAL-treated (A), ZL SAL-treated (B), ZN OVA-treated (C) and ZL OVA-treated (D) mice. Note the substantially decreased levels of Zinquin fluorescence in the OVA-treated mice tissues when compared with the SAL-treated tissues. Figure 3A shows a

typical profile line drawn across the epithelium along which fluorescence values were determined for 15 equally spaced intervals. Of interest is the apical distribution of fluorescence in the SAL-treated mice and the homogenous distribution in the OVA-treated mice (Figure 3).

Pro-caspase-3 Protein Levels

There was an increase in apical pro-caspase-3 levels from an average of 207.8 ± 5.2 GSU in the SAL-treated mice to 240.5 ± 2.2 GSU in the OVA-treated mice ($P \leq 0.001$). Increase in pro-caspase-3 also occurred in the basal compartments (Figure 3).

There was also a significant decrease in apical pro-caspase-3 levels in the airway epithelium of the ZL SAL-treated mice, which had a level of 190.0 ± 4.6 GSU compared with 207.8 ± 5.2 GSU in the ZN SAL-treated mice ($P \leq 0.05$, Figure 3A). Dietary Zn-related changes were greatest in the OVA-treated mice, occurring in both the apical and basal compartments (Figure 3). This protein has a largely apical distribution in the SAL-treated ZN mice (E), similar to the distribution of available Zn (A). This is illustrated in I, where sections were dual-labeled and the Zinquin fluorescence has been merged with that of pro-caspase-3 using image analysis. A similar pattern was seen in the SAL-treated ZL mice (B, F, and J). However, the OVA-treated ZN mice (C, G, and K) and the OVA-treated ZL mice (D, H, and L) had a more homogenous and disperse distribution. In the OVA-treated mice (K and L), the remaining Zn remains co-localized with pro-caspase-3, although the distribution of both was more homogenous.

Early Markers of Apoptosis

Figure 4 shows images of two early markers of apoptosis, active caspase-3 (with anti-17 kD caspase-3 antibody) and cleaved CK18 fluorescence labeling (with mouse anti-cleaved CK18) that have been merged from a collection of a z-series of optical confocal sections through individual tissue sections at 1- μ m intervals. Figures 4A and 4E are typical images showing very little labeling of either active caspase-3 or its cleaved substrate in ZN SAL-treated mouse airway epithelium. Similar results were seen for the ZL SAL-treated mice (B and F). This suggests little or no apoptosis in these tissues. By contrast, there was increased active caspase-3, particularly along the basement membrane (C) of the ZN OVA-treated mice, indicating enhanced apoptosis. Figures 4C and 4G also show some labeling in the subepithelial region and lamina propria. The significance of this labeling is unclear but may represent either eosinophil or fibroblast apoptosis.

In the ZL OVA-treated mice, substantial amounts of active caspase-3 and its cleaved substrate were seen in a localized pattern, particularly along the basement membrane (D and H) and also within the subepithelial region and lamina propria (*asterisk*). Note also a high frequency of apoptotic bodies at the luminal surface of the epithelium (*arrowed*) and in the lumen (H). Apoptotic bodies were enumerated under high magnification ($\times 2,500$) in 20 fields from duplicate slides for the different groups of mice. The mean number (\pm SD) of apoptotic bodies per high-powered field were 0.1 ± 0.4 for ZN SAL-treated

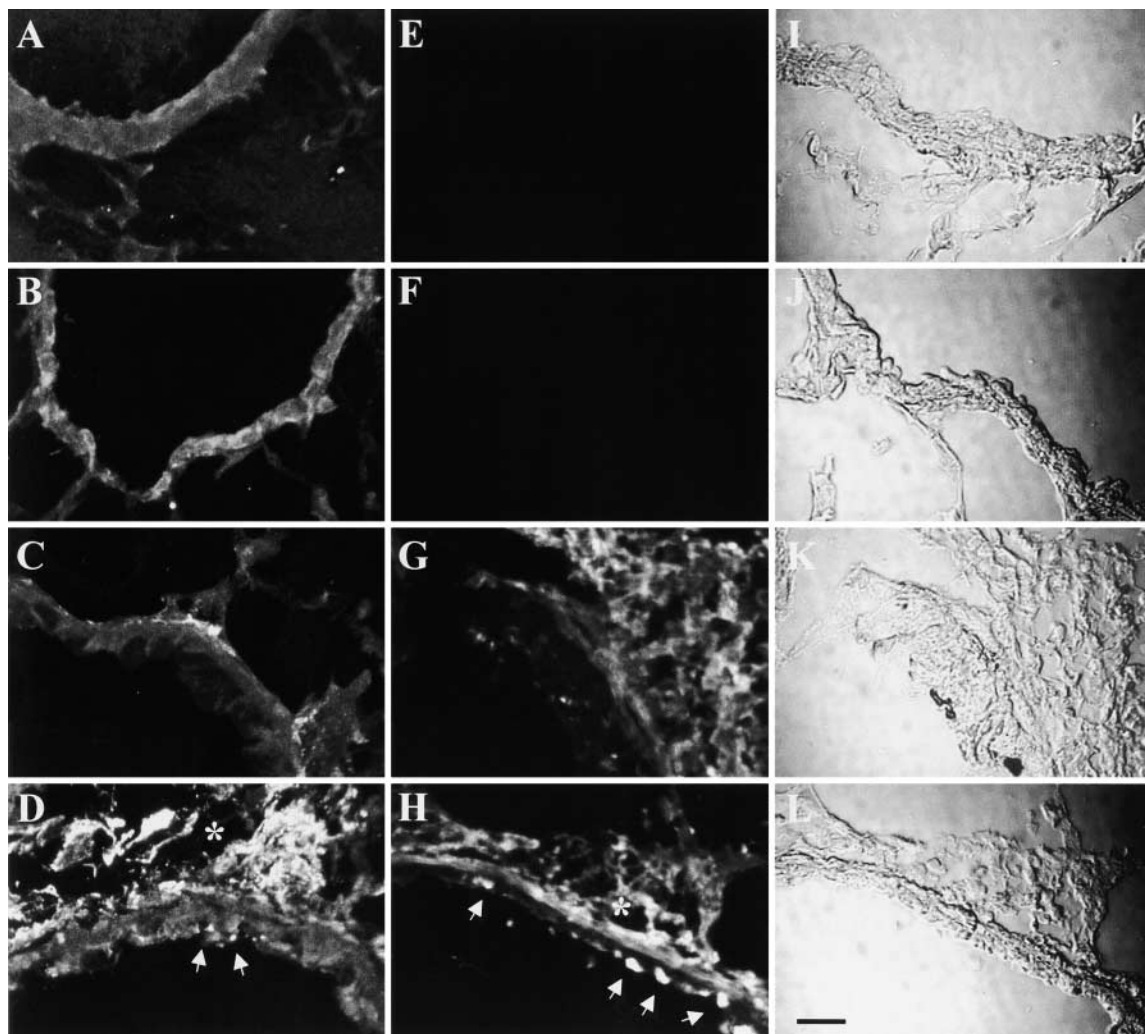


Figure 4. Comparison of activated caspase-3 and cleaved substrate cytokeratin 18 (CK18) in airway epithelium. Figure shows the labeling patterns for two early markers of apoptosis, the active form of caspase-3 (*A–D*) and the cleaved form of CK18, a caspase-3 substrate (*E–H*). For morphologic comparisons, the corresponding differential interference images of panels *E–H* are shown in panels *I–L*. ZN SAL-treated mice (*A* and *E*) had negligible apoptosis. There was a slight enhancement of active caspase-3 in the ZL SAL-treated mice (*B*) but no cleavage of CK18 was seen in the epithelium (*E*). A moderate increase in active caspase-3 labeling and cleaved CK18 were seen in the epithelium of ZN OVA-treated mice (*C* and *G*). However, there were marked increases in both active caspase-3 and cleaved CK18 in the epithelium of ZL OVA-treated mice (arrowed, *D* and *H*). Note also the positive staining within the subepithelial region and lamina propria (*). All images were taken with a $\times 40$ objective lens and $\times 3$ zoom. Each image is a merge of 12–16 optical slices of a z-series through the section. Typical images are shown and scale bar represents 20 μm .

mice and 0.3 ± 0.7 for ZL SAL-treated mice (not significant). However, there were significant increases in OVA-treated mice ($P \leq 0.005$), where ZN OVA-treated mice had 2.2 ± 1.7 apoptotic bodies and the ZL OVA-treated mice had 7.0 ± 3.3 ($P \leq 0.005$), suggesting that restriction of Zn further increases airway epithelial apoptosis over that due to the allergen treatment alone.

AHR

The ZN OVA-treated mice (Figure 5, filled triangles) showed a typical dose response to β -methacholine. AHR was significantly increased in the OVA-treated mice at all concentrations of β -methacholine from 6.25–50 mg/ml. For example, at 50 mg/ml of β -methacholine, AHR increased from $2.5 \pm$

0.1 penh units in the ZN SAL-treated mice to 4.8 ± 0.3 penh units in the ZN OVA-treated mice ($P \leq 0.05$).

There was a significant increase ($P \leq 0.05$) in AHR of mice given a ZL diet. This was best seen in the SAL-treated mice, where increases were seen at all concentrations of β -methacholine ≥ 12.5 mg/ml (Figure 5). With the ZL OVA-treated group, although all Penh readings were higher than the corresponding readings for the ZN OVA-treated groups, significance was only seen at 50 mg/ml β -methacholine ($P \leq 0.05$, Figure 5). Figure 5 shows data from a typical experiment. These findings were confirmed in each of three separate experiments. For example, the penh readings for the ZL SAL-treated mice, at 50 mg/ml dose of β -methacholine, were greater than those of the ZN SAL-treated mice by 52.9%

($n = 4$ mice), 40.4% ($n = 8$ mice), and 55.9% ($n = 8$ mice). The mean increase in AHR (50 mg/ml dose of β -methacholine) due to Zn deficiency alone was 49.7%, compared with an increase of 106.2% due to OVA treatment alone.

Discussion

In this study we have demonstrated an association between Zn deprivation and allergic airway inflammation. The major findings include (i) an increase in inflammatory indices (blood and tissue eosinophilia and mucus-secreting cell numbers) and AHR in Zn limited SAL-treated and OVA-treated mice; (ii) a reduction of Zinquin fluorescence which was associated with an increase in pro-caspase-3 labeling in the respiratory epithelium of OVA-treated mice; and (iii) an increase in active caspase-3 labeling in the basal region of the airway epithelium of OVA-treated mice, especially those which were Zn limited. These findings point to a hitherto unrecognized role for Zn in the respiratory system.

Our previous findings that primary sheep and pig respiratory epithelial cells are rich in available intracellular Zn, and that this is co-localized with pro-caspase-3 in the apical cytoplasm, suggest that this Zn may have a role in the survival of these cells. In support of this, Zn depletion enhanced oxyradical-induced caspase-3 activation and apoptosis, whereas Zn supplementation had a suppressive effect (10, 11). This paper takes these findings further by focusing on whether Zn influences the key physiologic (AHR) and histologic features (tissue and blood eosinophilia and mucus-secreting cell numbers) associated with asthma.

One of the main features of this study was the manipulation of Zn status via the diet to induce a mild Zn deficiency.

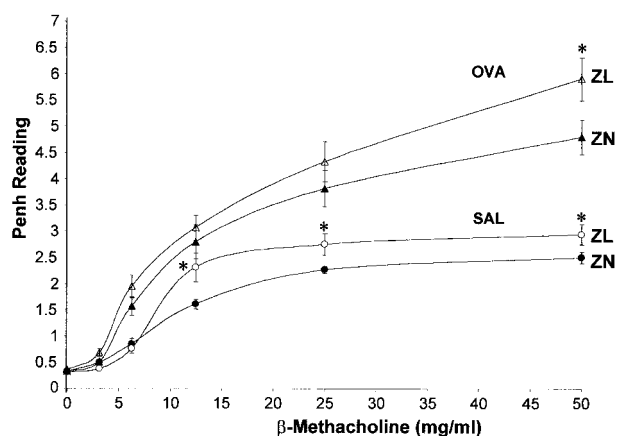


Figure 5. AHR to β -methacholine in Zn-manipulated, SAL-treated, and OVA-treated mice. Figure shows an expected increase in the AHR of OVA-treated mice (triangles) compared with the SAL-treated mice (circles). Importantly, there were significant increases in AHR in ZL mice (unfilled symbols) compared with ZN mice (filled symbols). For the SAL-treated ZL mice, there were significant increases in AHR at all concentrations above 6.25 mg/ml of β -methacholine. For the ZL OVA-treated mice, a significant increase was seen at the highest concentration (50 mg/ml β -methacholine). Values represent means \pm SEM of eight mice per group, Student's t test, $*P \leq 0.05$.

ciency. We achieved this by giving a ZL diet containing 14 mg/kg of Zn, rather than more commonly used diets which contained less than 1 mg/kg of Zn and therefore creating a more severe level of Zn deficiency (21). Zn deficiency was demonstrated by reduction in growth rates between Days 4 and 12, which was during the first period of OVA sensitization. Growth inhibition is a sensitive marker of Zn deficiency and the growth curves clearly show that Zn was the only limiting factor to weight gain in these mice. The rationale behind inducing a mild, rather than a more severe, Zn deficiency in these mice is that this more adequately reflects the suboptimal levels found in individuals with asthma (13–15, 17). This mild Zn deficiency caused significant differences in several inflammatory indices in our murine model.

In two human studies conducted in the 1990s, Schwartz and Weiss reported a negative relationship between wheezing and serum Zn:Cu ratios in a population study (18), whereas Soutar and colleagues noted an increase in the prevalence of atopy, bronchial reactivity, and risks of allergic symptoms in adults with the lowest intake of dietary Zn (19). Serum Zn and Cu levels are often negatively correlated (26). Because we have previously shown that Penh values proportionally reflect changes in resistance induced by spasmogens (23), the significant differences in Penh between experimental groups likely reflect functional changes in airflow within the airways. Our finding of increased AHR in ZL mice shows a direct effect of change in dietary Zn levels on airway function, which was approximately half of that observed due to allergen treatment. This suggests that even mild Zn deficiency has a significant influence on airway function. It is possible that the lesser effects of Zn deprivation on AHR in OVA-treated mice are due to the high level of inflammation already present in these mice, as seen in Table 2.

The mechanisms controlling the increased AHR in the ZL mice now need to be further investigated to determine whether this is an effect primarily on the smooth muscle or rather an effect on the airway epithelium. One possible mechanism of action is via inhibitory effects of Zn on muscarinic and nicotinic receptors (27, 28). One scenario is that Zn deprivation facilitates activation of muscarinic receptors, calcium channels, and calcium influx in smooth muscle cells, resulting in enhanced bronchoconstriction. Similar effects on airway epithelial cells may influence other factors. Alternatively, increased AHR in ZL mice may be due to the increase in the numbers of mucus-secreting cells noted in our ZL mice. Future studies will determine whether creating a more severe Zn deficiency in these mice further exacerbates AHR and whether incubation of airway smooth muscle cells with an exogenous Zn salt blocks β -methacholine-induced activation of muscarinic receptors *in vitro*. Other effects of Zn deficiency on AHR include enhanced inflammation due to shifts in the balance of lymphocyte subsets toward the proinflammatory Th₂ subset (29, 30), increases in activation of mast cells and eosinophils (31, 32), and increases in proinflammatory cytokines (33). The lack of effect of Zn deprivation on numbers of BALF eosinophils may relate to the severity of Zn deprivation created or to the kinetics of the response. This now needs to be investigated in mice receiving a broader range of Zn diets (inducing different levels

of Zn deficiency) and at different time points after induction of airway inflammation.

This study has provided the first information on lung epithelial Zn levels in allergic airway inflammation. One of the major findings of this study was the substantial loss of airway epithelial Zn in the lungs of the OVA-treated mice. The Zinquin fluorescence in the airway epithelium of the different groups of mice did not correlate with systemic levels of Zn in serum and liver as determined by AAS. This suggests that the mechanism of Zn loss is restricted to the airways. This may be due to one or more of the following mechanisms. The reduction in available Zn was homogenous and occurred in both the apical and basal compartments of the epithelium, which suggests that there is an overall loss of Zn from the epithelium rather than from one cell type. However, it must be formally determined whether this loss is predominantly in the ciliated cells, in the mucus cells, or in both, because in the OVA-treated mice ~ 70% of the airway epithelium consists of mucus-secreting cells. It is technically difficult to address this issue because Zinquin fluorescence can only be done on frozen tissues and not in conjunction with Alcian blue staining. One possible solution would be by a double-labeling technique using Zinquin fluorescence in conjunction with antibodies to specific epithelial cell markers such as muc5b.

One of the implications of Zn loss in the OVA-treated airway epithelium is the potential for enhanced apoptosis, because Zn is a potent suppressor of this mechanism of cell death. Zn is thought to act by blocking the activation of caspase-3 (34, 35). In light of this, the increase in pro-caspase-3 levels in the OVA-treated tissues is very interesting. The increased levels of pro-caspase-3 will render these cells more susceptible to apoptosis, which may be an important factor in the fragility of the epithelium in asthma. Apoptosis of bronchial epithelial cells in humans with asthma has recently been suggested by Benayoun and colleagues, who showed increased peroxisome proliferator-activated receptor γ levels in the lamina propria and the basement membrane (7). Our studies using antibody against active caspase-3 and its cleaved substrate cytokeratin 18 confirm these findings in a murine model of allergic airway inflammation, and show increased labeling at the basement membrane which may indicate that this is the first site of damage in the airway epithelium. Active caspase-3 was also increased in the lamina propria within regions where there is an accumulation of eosinophils. Whether this reflects direct apoptosis of eosinophils or damage to surrounding tissue fibroblasts by factors released from eosinophils is not known. The further increase in apoptosis in the epithelium of ZL OVA-treated mice is consistent with other studies showing enhanced rates of apoptosis in various tissues of Zn-deficient animals and humans (36, 37).

The mechanism behind the marked decrease in available Zn in the airway epithelium of OVA-treated mice is not known. This decrease in Zinquin fluorescence may represent a net reduction in epithelial Zn concentration and/or an incorporation of a greater proportion of Zn into metallothionein, which attenuates Zinquin fluorescence (24). If Zn has indeed been lost from the airway epithelium, several factors may play a part. First, losses of cellular Zn are

associated with enhanced cell turnover (26) a feature which is also found in individuals with asthma, in whom there is shedding of Zn-rich airway epithelial cells and eosinophils into the airway lumen. Second, Zn may be lost from the airway epithelial cells by secretion into the epithelial lining fluid or by binding to the negatively charged muco-polysaccharides in the mucin layer. Third, redistribution of Zn from certain tissues via the plasma to the liver is a common feature of inflammation (8), although this is unlikely, given our finding of a trend toward decreased liver Zn in our OVA-treated ZN mice, compared with SAL-treated mice. If the reduction of available Zn was due to a transfer of Zn to metallothionein within the airway epithelial cells, the resultant effect on caspase-3 activation would be similar to an absolute loss of this metal. Maret and colleagues have demonstrated that thionein, the apo-form of metallothionein, can remove Zn from inhibitory sites and reactivate several enzymes, including caspase-3 (38).

This study provides new data on the inter-relationship between Zn and airway epithelial cells, which may lead to a greater understanding of the association between Zn deficiency and airway disease. This provides an impetus for research to understand the molecular, biochemical, and cellular mechanisms for the possible beneficial roles of Zn in asthma.

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