# Attenuated Allergic Responses to House Dust Mite Antigen in Feed-Restricted Rats

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Caloric restriction has been shown to alter a broad range of immunological end points in both experimental animals and humans. The objective of this study was to investigate the effect of short-term moderate feed restriction (25% reduction) on allergic immune responses in Brown Norway rats. After 3 weeks of acclimation to their feed regimens, rats were sensitized and 2 weeks later challenged with house dust mite (HDM) antigen via intratracheal instillation. Feed restriction resulted in lower levels of antigen-specific IgE in serum and reduced antigen-specific lymphoproliferative activity in pulmonary lymph nodes. Feed restriction also attenuated pulmonary inflammation, as evidenced by lower levels of lactate dehydrogenase and total protein, decreased infiltration of neutrophils and eosinophils, and reduced secretion of pro-inflammatory cytokine tumor necrosis factor (TNF)-$\alpha$ in bronchoalveolar lavage fluid. In addition, feed restriction decreased TNF-$\alpha$ secretion in serum and decreased mRNA expression of TNF-$\alpha$ and interleukin-6 in pulmonary lymph nodes. We conclude that feed restriction strongly dampened the allergic immune responses to HDM in rats and that this attenuation was associated with decreased expression and secretion of pro-inflammatory cytokines. *Key words*: allergy, asthma, dust mites, eosinophil, feed restriction, IgE, immune response, inflammation, lung, T lymphocyte, tumor necrosis factor-$\alpha$. *Environ Health Perspect* 108:1125–1131 (2000). [Online 1 November 2000]
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Feed restriction studies in rodents show significant delays in aging-related degenerative diseases (*1*) and protection against cancer (*2*). It is also clear that feed restriction alters innate and specific immune function; however, its effects on allergic immune responses and resultant inflammation are not known.

The incidence of asthma has increased dramatically in the United States and other industrialized nations over the last 20 years. Changes in lifestyle may account for some of this increase. A recent epidemiologic study reported an association between being overweight (body mass index higher than 85th percentile) and the severity of asthma symptoms in susceptible children (*3*). An animal study showed that 18-hr fasting reduced ovalbumin-induced bronchoconstriction in guinea pigs (*4*). Other than these few reports, there is little information on feed restriction and allergic responses in the literature.

Inflammation is a hallmark of allergic asthma, and there is mounting evidence that feed restriction is anti-inflammatory. For example, long-term caloric restriction inhibits age-related increases in the serum pro-inflammatory cytokines tumor necrosis factor (TNF)-$\alpha$ and interleukin (IL)-6 in mice (*5*). We have recently demonstrated that short-term dietary restriction mitigates ozone-induced pulmonary inflammation in rats, and this is associated with lower levels of the pro-inflammatory cytokine IL-6 in bronchial alveolar lavage (BAL) fluid (*6*). We also showed that feed restriction improves antibacterial immune defenses in the lung (*7*) and that these effects were attributed to the increased phagocytic activity of alveolar macrophages. In another animal study, feed-restricted rats did not differ in T-cell mitogen responses compared to *ad libitum* controls but had decreased lympho-proliferative responses to recall antigens, suggesting that aspects of acquired (antigen-specific) immune function were impaired (*8*).

It is suggested that an imbalance of T-helper 1 (Th1) and Th2 cytokines plays an important role in allergic responses in animals (*9*) and in humans (*10*). Elevated levels of IL-4, IL-5, and IL-10 secretion by Th2 CD4+ cells, as well as suppressed levels of IL-2, and interferon (IFN-$\gamma$) secretion by Th1 CD4+ cells may enhance production of IgE, promote mast cell proliferation and maturation, augment airway hyperresponsiveness, and increase recruitment of eosinophils. Effects of feed restriction on the Th1 and Th2 responses have not been studied.

Our laboratory has developed a rat model of allergic responses to house dust mite (HDM) antigen and evaluated the impact of various chemical exposures on allergic responses to HDM (*11–13*). This model exhibits many of the hallmarks of allergic asthma such as increased HDM-specific IgE, elevated HDM-specific lymphoproliferative responses in lung-associated lymph nodes, antigen-induced bronchospasm, airway hyperresponsiveness, and pulmonary eosinophilia (*14*). We used this model to evaluate whether feed restriction can attenuate allergic responses to HDM antigen and whether this is accompanied by changes in pro-inflammatory cytokines levels in the pulmonary system.

## Materials and Methods

*Animals and dietary regimen.* Ten-week-old female Brown Norway rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN), housed two per cage in an environmentally controlled room with a 12-hr light/dark cycle, and fed Laboratory Rodent Diet 5001 (Purina Mills, St. Louis, MO). Randomly selected animals were tested serologically upon arrival and monitored throughout the study to ensure that rats were free of Sendai virus, murine pneumonia virus, a variety of other rodent viruses, as well as *Mycoplasma* sp. Rats were also monitored for and found to be free of ectoparasites and endoparasites. All procedures were approved by the Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, before commencing the study.

After a 1-week acclimation period, the dietary treatments were initiated as follows. Average feed consumption by animals fed *ad libitum* was determined daily. This value was then multiplied by 0.75, and the resultant mass of food was presented to the restricted animals the next morning. The restricted animals always ate the entire portion presented. The dietary treatment began 3 weeks before antigen exposure and continued throughout

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the experiment. We monitored body weights every other day throughout the study. All nutrients provided by the dietary regimens exceeded levels recommended by the National Research Council (*15*).

*Antigen sensitization and challenge procedure.* Semipurified extracts of *Dermatophagoides farinae* and *D. pteronyssinus* (Greer Laboratories, Lenoir, NC) were rehydrated in sterile saline to 10 mg/mL (containing 172 mg HDM group I antigen/mg dry weight), mixed, and stored in aliquots at –70°C. After 3 weeks of acclimation of their respective dietary regimens, the rats were lightly anesthetized by halothane (Aldrich, Milwaukee, WI) inhalation and sensitized with a single intratracheal instillation of 17.6 μg of HDM group I antigen in 0.3 mL of sterile saline. Two weeks later, rats were challenged by the identical procedure.

*Experimental design.* Rats were acclimated for 1 week before dietary treatment and randomly divided into two groups, *ad libitum*-fed (control) and feed restricted, with 20 rats for each group. At each of the following four time points, 5 animals per group were euthanized by sodium pentobarbital overdose (180 mg/kg, intraperitoneal injection): 7 days postsensitization, 14 days postsensitization, 2 days postchallenge, and 7 days postchallenge. We obtained samples at each time point. Serum was collected through cardiac puncture, and bronchoalveolar lavage (BAL) fluid was collected after lavage with a single volume of saline (35 mL/kg) via tracheal cannula. The spleen and pulmonary lymph nodes were also collected. We analyzed serum samples for HDM-specific IgE, IgG, and IgA. In addition, differential cell counts were performed on BAL cells and BAL fluid was analyzed for lactate dehydrogenase (LDH), total protein, eosinophil peroxidase (EPO), IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α. The lymphoproliferative responses of cells from spleen and pulmonary lymph nodes were assessed. We also determined cytokine gene expression in cells from pulmonary lymph nodes. Data reported are representative of three separate experiments.

*Biochemical and cytological analyses.* Cell counts in BAL fluid were obtained with a hemocytometer, and cell viability was assessed by trypan blue dye exclusion. After dilution, 50,000 cells from each sample were centrifuged onto microscope slides using a Cytospin Model II (Shandon, Pittsburgh, PA) and stained with Diff Quick (American Scientific, Sewickley, PA). We performed differential counts in duplicate on 200 cells per specimen for identification of alveolar macrophages, polymorphonuclear leukocytes (PMN), lymphocytes, and eosinophils.

After centrifugation at 425*g* for 10 min at 4°C, we assessed supernatant BAL fluid for LDH using a commercially available method (Sigma, St. Louis, MO). Total protein in BAL fluid was detected with Pierce Coomassie Plus protein assay reagent (Pierce, Rockford, IL) using bovine serum albumin (BSA; Sigma) as a standard. EPO, as a measure of eosinophil activation, was determined using the OPD Tablet Set (Sigma). All biochemical assays were modified for automated analysis by a Cobas Fara II centrifugal spectrophotometer (Hoffman-LaRoche, Branchburg, NJ).

*Antibody analysis.* We assessed serum and BAL fluid for HDM-specific immunoglobulins by ELISA. Before the assay, pooled serum and BAL samples were tested to determine appropriate dilution factors for the linear range of the reaction curve. For the assay, 96-well polystyrene plates (Costar, Cambridge, MA) were coated overnight with 17.6 μg semipurified HDM/mL of 0.05 M carbonate buffer (pH 9.6) at 4°C. The plates were washed five times with 0.1 M phosphate-buffered saline (PBS) containing 0.05% Tween 20. Each well was filled with a blocking solution of 1% BSA in PBS and incubated for 2 hr before washing. Diluted serum samples (1:100 for IgG, 1:10 for IgE and IgA) or BAL fluid (1:10 IgG, 1:2 for IgE and IgA) were added to the wells and incubated overnight with the coated HDM at 4°C. Following another wash step, biotin-labeled mouse anti-rat IgG, IgE, or IgA (Accurate, Westbury, NY) was added to the plates at optimal dilutions and incubated for 2 hr at room temperature. We removed excess antibody by washing, and added horse radish peroxidase-streptavidin (Vector Laboratories, Burlingame, CA) to the plates, which were then incubated for another 2 hr at room temperature. A solution of TM blue enzyme reagent (TSI-CDP, Milford, MA) was added and the color change was measured at a wavelength of 650 nm with an ELISA plate reader (Molecular Devices, Menlo Park, CA). We subtracted background values from all sample values to correct for nonspecific binding effects. The ELISA assay was optimized using a checkerboard titration to provide optimal concentrations of antigen and antibodies for the reaction. We compared reaction times and optical density (OD) values to historical data obtained during the development of this assay. Other experiments from our laboratory (*14*) have shown good concordance between the IgE ELISA assay and antigen-specific passive cutaneous anaphylaxis (PCA) tests.

*Lymphoproliferative responses.* Spleens and lung-associated lymph nodes from the mainstem bronchus were collected and gently homogenized in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mM Hepes buffer, $5 \times 10^{-5}$ M 2-mercaptoethanol, 100 units/mL penicillin, and 100 mg/mL streptomycin (GIBCO, Grand Island, NY). After three lysing steps with 0.89% $NH_4Cl$ and RPMI medium, cells were washed, counted with a hemocytometer, and resuspended to a concentration of $5 \times 10^6$ cells/mL. We assessed cell viability by trypan blue dye exclusion. We added 100 μL aliquots of each cell suspension in triplicate to wells of 96-well, flat-bottomed tissue culture plates. Cells were incubated in the presence of 1.76 μg semipurified HDM or medium alone for 4 days. Preliminary studies indicated that these were optimal culture conditions. Eighteen hours before the end of the incubation period, 0.5 μCi [$^3$H]thymidine (DuPont, Wilminton, DE) in 20 μL RPMI medium was added to each well. Cells were harvested automatically onto filters (Skatron, Sterling, VA) and the [$^3$H]thymidine incorporation was measured by scintillation (Packard, Meriden, CT). Lymphocyte responsiveness was expressed as the difference in radiotracer counts between cells incubated with HDM (stimulated) and those incubated with medium alone (unstimulated).

*Cytokine assays.* We determined TNF-α, IL-1β, IL-6, IFN-γ, IL-2, IL-4, and IL-10 secretions in serum and in BAL fluid according to the manufacturer's procedure using commercial ELISA kits from BioSource (Camarillo, CA).

*Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR).* We isolated cells from lung-associated lymph nodes during lymphocyte proliferation assay and pooled them according to treatment. About $5 \times 10^6$ cells from each treatment group were homogenized in 1 mL Tri Reagent (Sigma), and total cellular RNA was extracted according to the manufacturer's procedure. To synthesize complementary
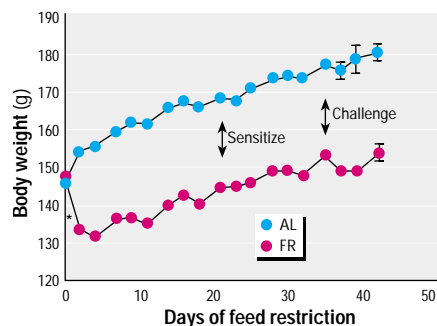


**Figure 1.** Effect of feed restriction on body weight of house dust mite-exposed Brown Norway rats. Abbreviations: AL, *ad libitum*; FR, feed restricted. Body weight of experimental animals was monitored 3 days/week during the experimental period. All animals were sensitized and challenged on days 21 and 35, respectively, as indicated, and 5 rats from each dietary group were sacrificed 7 and 14 days postsensitization and 2 and 7 days postchallenge. Data are means ± SE (*n* = 5).
*Significant difference ($p < 0.05$) between AL and FR groups at day 2 and thereafter (multivariate ANOVA).

DNA, 1.0 µg of RNA was resuspended in a 20-µL final volume of the reaction buffer (50 µM Tris-HCL, 75 mM KCl, 10 mM dithiothreitol, 3.0 mM MgCl$_2$, 10 mM of each deoxynucleotide triphosphate, 1 U/µL of RNase inhibitor, pH 8.3; Perkin-Elmer Cetus, Foster City, CA) containing 0.5 µg oligo d(T) 12-18 primer (GIBCO BRL, Gaithersburg, MD). When the reaction mixture reached 42°C, 200 U SuperScript II reverse transcriptase (GIBCO BRL) was added into each tube, incubated for 30 min at 42°C, and stopped by denaturing the enzyme at 99°C for 5 min. The reaction mixture was diluted with distilled water to 50 µL. PCR primers for rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH), IL-1, IL-6, TNF-α, IL-2, and IFN-γ were purchased commercially from Clontech (Palo Alto, CA); PCR primers for rat IL-4 and IL-5 were synthesized by the core facility of the Human Studies Division at the U.S. EPA (Chapel Hill, NC). The sequences of the primers for rat IL-4 and IL-5 were as follows: *a*) IL-4 (sense: 5´-TGATGGGTCTCA-GCCCCCACCTTGC-3´, anti-sense: 5´-CTTTCAGTGTTGTGAGCGTGG-ACTC-3´); and *b*) IL-5 (sense: 5´-TGAC-GAGCAATGAGACGATGT-3´, anti-sense: 5´-TCATCACGCCAAGGAACTCT-3´).

We added 5-µL aliquots of the synthesized cDNA to 45 µL of the PCR mixture containing 1.5 µl of MgCl$_2$ (0.2 mM), 5 µl of 10 × PCR buffer (20 mM Tris HCl, 50 mM KCl, pH 8.4), 1 µL deoxynucleotides (0.2 mM each), 1 µL of sense and anti-sense primers (0.5 µL each, 0.2 mM), 0.3 µL Taq DNA polymerase (Promega, Madison, WI), and 36.2 µL of RNase-free water. The amplification was initiated by 1 min of denaturation at 95°C for 1 cycle, followed by 20–35 cycles at 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec using a GeneAmp PCR System 9600 DNA Thermal Cycler (Perkin-Elmer Cetus). After the last cycle of amplification, the samples were incubated at 72°C for 7 min. For each set of primers, dilutions of cDNA were amplified for 20, 25, 30, and 35 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength.

Amplified PCR products along with the molecular weight marker, 100 bp DNA ladder (GIBCO BRL), were separated electrophoretically (2% agarose gel at 75 V for 60 min) and visualized by ultraviolet illumination after staining with 0.5 µg/mL ethidium bromide. Gels were photographed with Type 55 positive/negative film (Polaroid, Cambridge, MA). We determined the relative changes in mRNA transcripts and performed the densitometric analysis of the captured image using the AlphaImage 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA). The area under the curve was normalized against G3PDH content.

***Statistical analysis.*** We analyzed the data for all outcome variables, except body weights, using a two-way analysis of variance (ANOVA) model. The two independent effects were food consumption at two levels, *ad libitum* and restricted, and time after sensitization or challenge at four levels, 7 days postssensitization, 14 days postsensitization, 2 days postchallenge, and 7 days postchallenge. After identification of significant main effects or interactions, we performed pairwise comparisons among combinations of the various independent variables.

We analyzed the time course of body weight using multivariate ANOVA. The single independent variable was food consumption at two levels, *ad libitum* and restricted, and the dependent variable was animal
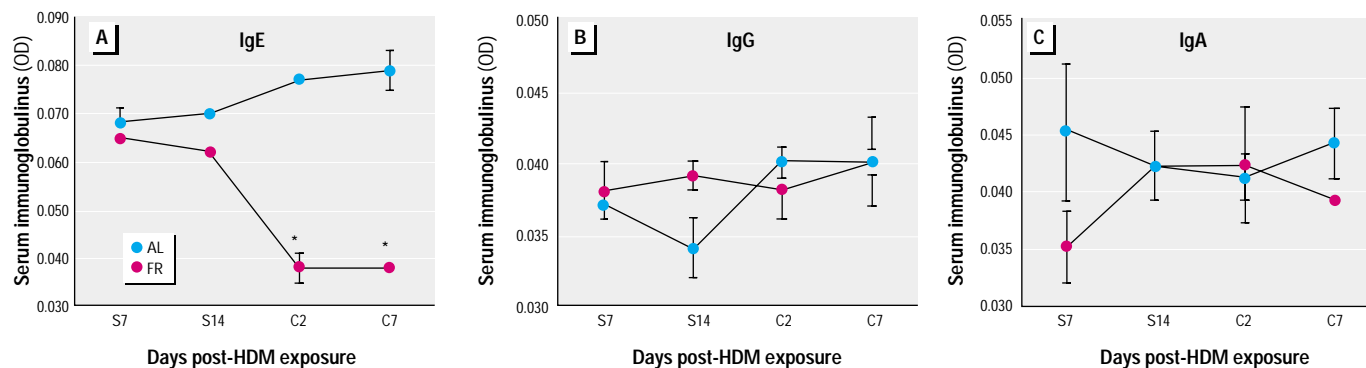


**Figure 2.** Effect of feed restriction on house dust mite-specific antibodies in serum. Abbreviations: AL, *ad libitum*; C2, 2 days postchallenge; C7, 7 days postchallenge; FR, feed restricted; S7, 7 days postsensitization; S14, 14 days postsensitization. Sera were collected at the indicated times and analyzed by ELISA. Data are means ± SE (*n* = 5) of optical density (OD). Two-way ANOVA was applied, and pairwise comparisons were performed following a significant interaction. *Significant difference (*p* < 0.05) between AL and FR groups.
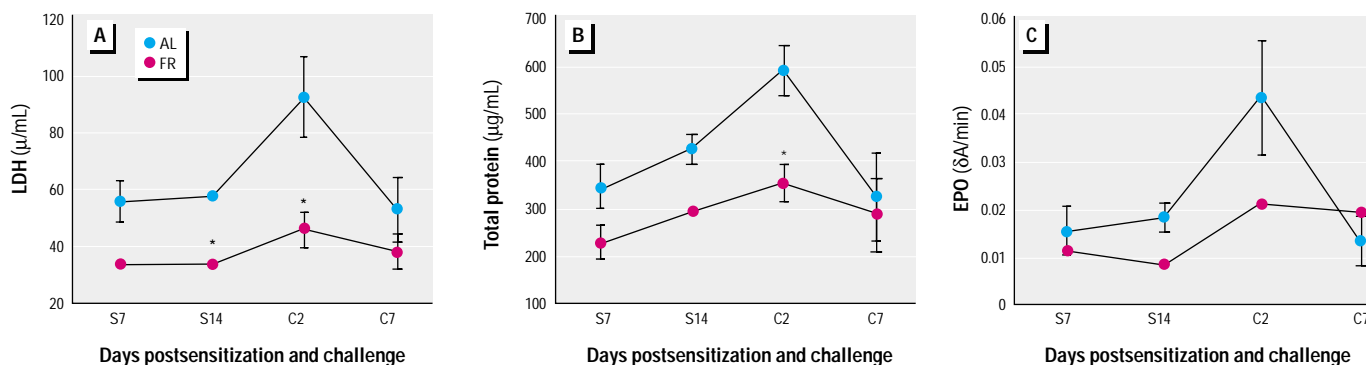


**Figure 3.** Effect of feed restriction on LDH, total protein, and EPO in BAL fluid. Abbreviations: AL, *ad libitum*; C2, 2 days postchallenge; C7, 7 days postchallenge; FR, feed restricted; S7, 7 days postsensitization; S14, 14 days postsensitization. BAL fluid (35 mL/kg) was collected at the indicated times and centrifuged at 1,500 rpm for 10 min at 4°C. Supernatant was analyzed. Data are means ± SE (*n* = 5). δA denotes changes in absorption. Two-way ANOVA was applied, and pairwise comparisons were performed following a significant interaction. *Significant difference (*p* < 0.05) between AL and FR groups.

weight. We performed comparisons to the initial weight to assess whether a subsequent weight difference, attributable to feeding regimens, had appeared.

The level of significance was set at 0.05. We made appropriate multiple comparison adjustments to preserve the experiment-wise error rate.

## Results

Two physiologically distinctive groups of rats were created by imposing a moderate feed restriction (25% of the amount consumed by *ad libitum* group) on one group of rats. *Ad libitum*-fed rats grew steadily at about 0.8 g a day, as indicated by their body weight (Figure 1). After a weight loss of 15.8 g in the first 4 days, the feed-restricted rats also grew steadily during the rest of the experimental period at about the same rate as *ad libitum* group. However, the weight gap caused by feed restriction persisted in feed-restricted animals.

To assess the effect of feed restriction on allergic immune responses to HDM, allergen-specific IgE was measured in the serum (Figure 2). Equivalent levels of HDM-specific IgE were found in *ad libitum* and feed-restricted groups after sensitization. After antigen challenge, however, the HDM-specific IgE increased in the *ad libitum* group but decreased by 50% in the feed-restricted group compared to levels before antigen challenge. No differences in the level of serum HDM-specific IgG or IgA were found between the two groups at any time points measured.

Both *ad libitum*-fed and feed-restricted rats were instilled with 0.3 mL of HDM antigen. Because feed-restricted rats were lighter in body weight, they might have received more antigen per milligram of lung tissue than *ad libitum*-fed rats, assuming lung size correlates positively with body weight. This possibility does not adversely affect the interpretation of the experimental results, because feed-restricted rats exhibited less allergic responses to HDM antigen despite the fact that they might have received more antigen per milligram of lung than *ad libitum*-fed rats.

Levels of LDH, total protein, and EPO in BAL fluid were measured to evaluate pulmonary cell membrane integrity, edema, and eosinophil activation, respectively. The dynamics of these biochemical analyses revealed that the overall levels of LDH, total protein, and EPO appeared lower in the feed-restricted group compared to the *ad libitum* group. There were increases in LDH, total protein, and EPO levels 2 days after HDM challenge in *ad libitum* rats compared to levels before antigen challenge (Figure 3); however, such responses were sharply attenuated in feed-restricted rats.

Pulmonary allergic inflammation in response to HDM was examined by differential cell count of BAL fluid. The number of eosinophils, neutrophils, macrophages, and lymphocytes in *ad libitum* rats increased dramatically 2 days after HDM challenge (Figure 4). In contrast, feed-restricted rats showed no such cellular influx, although there was a significant increase in the number of BAL macrophages in these animals 14 days after HDM sensitization.

Equivalent levels of lymphocyte proliferation were found in *ad libitum* and feed-restricted groups after antigen sensitization.

After antigen challenge, however, lymphoproliferative responses increased after HDM challenge in *ad libitum* rats (Figure 5) but were only modestly higher in the feed-restricted animals. Lymphocyte proliferation of spleen cells in response to HDM was similar between the two groups at all time points measured.

The secretion of the pro-inflammatory cytokine TNF-α in serum and BAL fluid differed between two dietary groups. Compared to levels before antigen challenge, levels of TNF-α were significantly increased 2 days
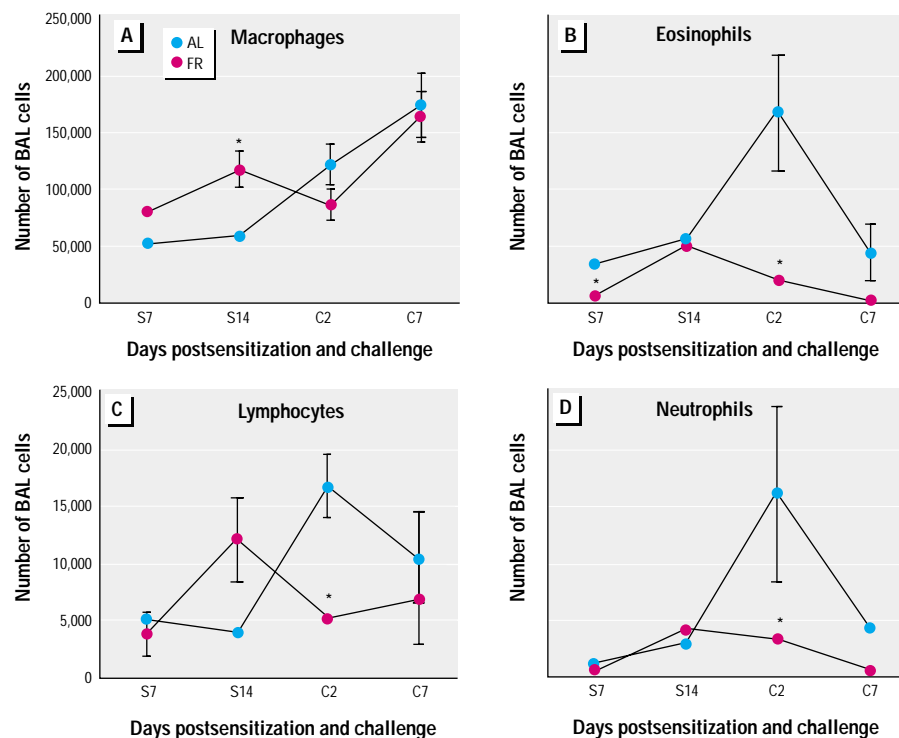


**Figure 4.** Effect of feed restriction on BAL cell differential. Abbreviations: AL, *ad libitum*; C2, 2 days postchallenge; C7, 7 days postchallenge; FR, feed restricted; S7, 7 days postsensitization; S14, 14 days postsensitization. Data are means ± SE (*n* = 5) of number of cells per animal. Two-way ANOVA was applied, and pairwise comparisons were performed following a significant interaction.
*Significant difference (*p* < 0.05) between AL and FR groups.



**Figure 5.** Effect of feed restriction on house dust mite-specific lymphocyte proliferation in the spleen and pulmonary lymph nodes. Abbreviations: AL, *ad libitum*; C2, 2 days postchallenge; C7, 7 days postchallenge; dpm, disintegrations per minute; FR, feed restricted; S7, 7 days postsensitization; S14, 14 days postsensitization. Cells were collected at the indicated times and cultured in the presence or absence of HDM. Data are means ± SE (*n* = 5). Two-way ANOVA was applied, and pairwise comparisons were performed following a significant interaction.
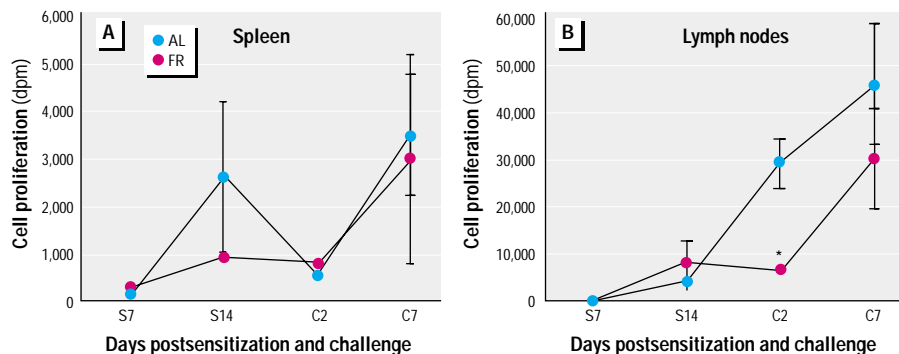*Significant difference (*p* < 0.05) between AL and FR groups.

after HDM challenge in both serum and BAL fluid of the *ad libitum* group, and the magnitude of the increase was higher in BAL than in serum (Figure 6). Such an increase was not found in serum and was significantly reduced in BAL fluid from feed-restricted rats. The secretion of IL-1β and IL-6 was undetectable in both serum and BAL fluid in all animals (data not shown).

The mRNA levels of pro-inflammatory cytokines TNF-α and IL-6 in preparations from pooled pulmonary lymph nodes appeared to be lower in the feed-restricted group compared to the *ad libitum* group 7 days after HDM sensitization (Figure 7). After challenge, however, mRNA levels increased and were equivalent between control and feed-restricted rats. Unlike IL-6 and TNF-α expression, no differential responses in IL-1 expression were apparent between the *ad libitum* and feed-restricted groups.

To investigate the effect of feed restriction on the balance of Th1 and Th2 cytokines, the protein levels of IL-2, IFNγ, IL-4, and IL-10 in serum and in BAL fluid as well as the mRNA levels of IL-2, IFNγ, IL-4, and IL-5 in pulmonary lymph nodes were assessed. No detectable levels of IL-2, IFNγ, IL-4, or IL-10 were found in serum or in BAL fluid, and there were no differences between *ad libitum* and feed-restricted groups in mRNA of cytokines detected (data not shown).
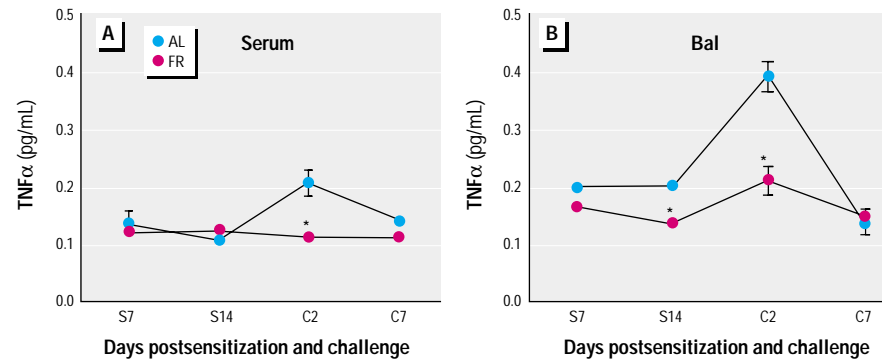
## Discussion

Several studies have shown that feed restriction can alter immune responses to experimental antigens (*8,16*) and may have profound effects
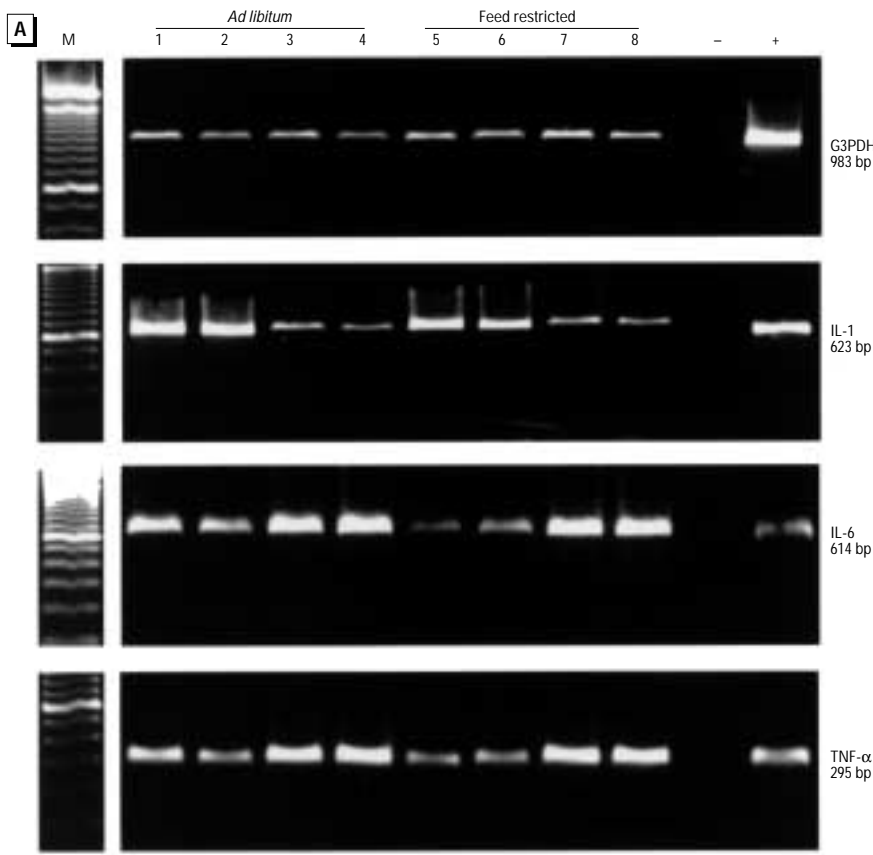
**Figure 6.** Effect of feed restriction on TNF-α in serum and BAL fluid. Abbreviations: AL, *ad libitum*; C2, 2 days postchallenge; C7, 7 days postchallenge; FR, feed restricted; S7, 7 days postsensitization; S14, 14 days postsensitization. Data are means ± SE ($n$ = 5). Two-way ANOVA was applied, and pairwise comparisons were performed following a significant interaction.
*Significant difference ($p$ < 0.05) between AL and FR groups.

**Figure 7.** (*A*) Effect of feed restriction on IL-1, IL-6, and TNF-a mRNA in pulmonary lymph nodes. Abbreviations: AL, *ad libitum*; C2, 2 days postchallenge; C7, 7 days postchallenge; FR, feed restricted; M, molecular weight marker; S7, 7 days postsensitization; S14, 14 days postsensitization. Total RNA was extracted from pooled pulmonary lymph node cells (5 × 10^6) at S7, S14, C2, and C7, and subsequently analyzed by RT-PCR. M, 100 bp DNA ladder; lanes 1–4 = S7, S14, C2, and C7, respectively, in *ad libitum* group; lanes 5–8 = S7, S14, C2, and C7, respectively, in feed-restricted group; lanes – and + represent negative (water) and positive controls, respectively, for each of the primers used. Loading equivalence was verified by equal intensities of G3PDH mRNA. The expected sizes of the PCR products are 983 bp for G3PDH, 623 bp for IL-1, 614 bp for IL-6, and 295 bp for TNF-α. Relative changes in (*B*) IL-1, (*C*) IL-6, and (*D*) TNF-α mRNA. The video image intensities of IL-1, IL-6, and TNF-α mRNA transcripts in (*A*) were determined using an AlphaImager 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA) and are expressed in arbitrary units. Densitometric analysis of the captured image was performed using computer software attached to the system. The area under the curve for IL-1, IL-6, or TNF-α was normalized against corresponding G3PDH contents.

on antioxidant balance in various tissues (*17*). The purpose of this study was to determine if feed restriction affected immunologic sensitization to HDM antigen in Brown Norway rats and if the dietary restriction could alter the outcome of allergic disease induced after a subsequent antigen challenge. We found that feed restriction did not affect initial antigen priming: no changes in lymphocyte proliferative responses and antigen-specific antibody production were evident. However, after antigen challenge, the boosting of immune function and the inflammatory influx seen in the lungs of *ad libitum*-fed animals was significantly abrogated in feed-restricted rats. Specifically, these animals had decreased levels of HDM-specific IgE in serum, lower HDM-specific lymphocyte proliferation in pulmonary lymph nodes, reduced infiltration of eosinophils into the lung, and lower levels of LDH and protein in the BAL fluid.

It is unlikely that a general reduction in lymphocyte reactivity accounted for these observations because several studies have shown that moderate feed restriction (of the magnitude used on our studies) actually enhances T- and B-cell mitogen responses, natural killer cell function, and macrophage function in mice and rats (*7,8*). Rather, it appears that acquired (antigen-specific) immune responses were reduced and that these effects were associated with decreased immune-mediated lung disease.

Although the mechanisms for this attenuation are unclear, reduced initial expression of the pro-inflammatory cytokines TNF-α and IL-6 was associated with decreased allergic responses to HDM in feed-restricted animals. TNF-α is an important mediator in human allergic reactions (*18*). Numerous studies have demonstrated that TNF-α upregulates intrapulmonary expression of ICAM-1 (intercellular adhesion molecule-1) (*19*), enhances adhesion of activated eosinophils to respiratory epithelial cells (*20*), and mediates recruitment of both neutrophils and eosinophils during airway inflammation (*21*). Studies also show that TNF-α is necessary for eosinophil transendothelial migration (*22*). In one particular study using Brown Norway rats, a dose-dependent inhibition of neutrophil and eosinophil infiltration into BAL fluid was observed when TNF-α receptors were blocked by Ro 45-2081 (*23*).

After antigen challenge, TNF-α levels were consistently lower in both the serum and BAL fluid of feed-restricted animals. mRNA expression of this cytokine and IL-6, however, was increased after challenge and was equivalent between dietary groups. Quite possibly, the RT-PCR reactions from tissue extracted at these time points were saturated compared to baseline levels. Alternatively, cytokine expression may have been less affected in the lymph nodes compared to lung tissue, where differences in antioxidant levels and mRNA expression of TNF-α are known to exist between *ad libitum* and feed-restricted animals (*6,7*).

Two possible mechanisms for the decreased immune responses and allergic lung disease in feed-restricted rats are overproduction of corticosteroids and reduced oxidative stress. Dietary restriction is associated with diurnal hypercorticism in rodents (*24*), which in turn reduces pro-inflammatory cytokine production (*25–28*) and inhibits expression of adhesion molecules (*29*). Furthermore, glucocorticoids suppress IL-5 and granulocyte macrophage–colony-stimulating factor production and eosinophilia in nasal and pulmonary tissues (*30,31*). Dietary restriction also resets the oxidant status in tissues and organs via a combination of decreased free-radical production and increased levels of antioxidants (*17*). Because overproduction of oxidants by leukocytes plays an important role in inflammatory lung disease and asthma, it is reasonable to expect that reduced free-radical production would lead to decreased tissue damage during an inflammatory event.

Although we propose hypercorticism and reduced oxidative stress to be two separate plausible mechanisms by which feed restriction might attenuate immune and inflammatory responses, their cellular and molecular interactions are, in fact, closely related. Glucocorticoids reduce glucose uptake and energy metabolism in peripheral tissues, which subsequently decreases the rate of intracellular glycoxidation, thus mitigating oxidative stress through reduced production of mitochondrial free-radicals. Increased glucocorticoids and decreased free-radical output reduce the expression of nuclear transcription factors AP-1 (activation protein-1) and NFκB (nuclear factor kappa B) (*32*), which regulate the expression of pro-inflammatory cytokines, chemokines, and adhesion molecules. The combination of altered transcriptional activity of immunoregulatory cytokines and differences in oxidant status between *ad libitum*-fed and feed-restricted rats provides a rational explanation for differences in the severity of inflammatory lung disease between these two different treatment groups. Although the mechanisms for this effect are still largely unknown and warrant more exploration, our results indicate that nutritional status should be considered as an important feature when modeling human disease in experimental systems and that unrestricted caloric intake may be a risk factor in immune-mediated lung diseases such as asthma.

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