Effect of L-Tryptophan Supplementation on Eosinophils and Eotaxin in Guinea Pigs

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Eosinophilia Myalgia Syndrome is a hypereosinophilic disorder that appears to result from the ingestion of the dietary supplement L-tryptophan by susceptible individuals. It is unclear if this disease results from tryptophan, contaminants found in tryptophan, individual predisposition (such as immune status and allergies), or some combination of effects. To evaluate effects of L-tryptophan on eosinophil migration, guinea pigs were compared with or without supplemental tryptophan (0.4 g/kg/day), with or without immune sensitization, and with or without immune challenge. Eosinophil counts were obtained from bone marrow, blood, lung, and bronchial alveolar lavage fluid (BAL). Lung cells were obtained to measure eotaxin concentrations in supernates and lysates with or without antigen and calcium ionophore challenge using direct ELISA. Skin biopsies were taken from both non-injected and antigen injection sites. The tryptophan supplemented, antigen-sensitized/antigen-challenged guinea pigs showed a significant decrease in blood eosinophils, compared to control (cellulose) supplemented antigen-sensitized/antigen-challenged guinea pigs [$(0.086 \pm 0.023) \times 10^6$ vs $(0.147 \pm 0.021) \times 10^6$ eosinophils/ml recovered, respectively] with a significant increase in BAL eosinophils [$(0.052 \pm 0.008) \times 10^6$ vs $(0.033 \pm 0.005) \times 10^6$ eosinophils/ml recovered, respectively]. Unchallenged lung cell lysates from tryptophan-supplemented guinea pigs contained significantly less eotaxin compared to cellulose-supplemented guinea pigs regardless of whether they were sensitized (0.006 ± 0.002 vs 0.027 ± 0.008 ng/10^6 cells, respectively). No differences were observed in skin biopsies between cellulose and tryptophan groups. These results suggest that L-tryptophan-supplemented guinea pigs have altered eotaxin regulation, a potential mechanism by which human overconsumption of tryptophan dietary supplements could lead to hypereosinophilic disorders in susceptible individuals. [Exp Biol Med Vol. 226(3):177–184, 2001]

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L-tryptophan (T) is an essential dietary aromatic amino acid which is catabolized by two distinct pathways. One metabolic pathway results in kynurenine (KYN), quinolinic acid (QA), and ultimately NAD and NADP, while the other pathway (metabolizing ~2–5% of dietary T) leads to the production of serotonin (1). The kynurenine pathway is regulated primarily by interferon gamma (IFNγ) induction of the rate limiting enzyme indoleamine-2,3-dioxygenase (IDO) (2, 3). Because T is a precursor of serotonin, T supplementation has been recommended for individuals suffering from a variety of conditions associated with decreased serotonin and T levels including sleep disorders, fibromyalgias, chronic fatigue syndrome, and depression.

In 1989, eosinophilia-myalgia syndrome (EMS) (characterized by peripheral eosinophilia, severe myalgia, dyspnea, pneumonitis, and skin sensitivity) was first described, and epidemiologic studies linked the disorder to ingestion of large doses of dietary T supplements (4, 5). Traceback studies linked a specific Japanese manufacturer's (Showa Denko KK, Japan) product to nearly all of the individuals diagnosed with EMS (6). Analysis of this Showa Denko T (manufactured after a change in production practices in 1988) detected various contaminants, some of which were subsequently suspected to be the cause of the syndrome (8–11). The traceback studies are disputed by some epidemiologists (12). In fact, the same epidemiologists that question the traceback studies wonder if EMS is related to tryptophan itself (12, 13) or if the EMS epidemic even occurred (14). Interestingly, while altered tryptophan metabolism has been described in EMS (15), it has been recognized for some time that altered T metabolism is also involved in scleroderma, eosinophilic fascitsis, and rheumatoid arthritis (16). To date, studies with animal models for the disease have been unsuccessful in manifesting the symptoms of EMS observed in humans. The results of these animal stud-

ies, unfortunately, have been inconclusive, contradictory, and have ultimately failed to demonstrate any clear relationship between Showa Denko T, its contaminants, and/or non-implicated T with abnormalities in experimental animals (6, 7).

Speculation has been ongoing as to the mechanism of this debilitating disease. Previous studies have not adequately addressed the following factors: (i) that preexisting immune activation might have influenced the development of the disease; (ii) the animal models chosen (rats and mice) have a non-eosinophilic nature; and (iii) over-ingestion of T alone or an interaction with T and the implicated contaminants could have been a factor (rather than the contaminants alone). Indeed, kynurenine pathway metabolites of T and IFNγ were increased in the serum of some of the EMS patients, suggesting that these patients had altered T metabolism (17). The increased levels of these metabolites of T returned to normal with steroid treatment, suggesting that the altered T metabolism was the result of IDO induction by IFNγ, not an inborn error of metabolism (18).

In an attempt to address these concerns, the following studies were conducted to evaluate the effect of consumption of T on eosinophil migration in the presence of immune activation. The guinea pig was selected because it is an accepted model for eosinophil migration, being naturally eosinophilic (19–22). Because skin is one of the most commonly affected organs in EMS (5), skin biopsies were taken to evaluate any inflammatory changes. Eosinophil counts were obtained from blood, bone marrow, lung, and bronchial alveolar lavage fluid (BAL). The tissues selected correspond to the route of eosinophil migration in the guinea pig in response to antigen challenge (23), and both blood and lungs were commonly affected in EMS. Immune activation was accomplished by an immune deviation sensitization protocol that produces high levels of IgG_1 (the main reaginic or homocytotropic antibody in guinea pigs). Eotaxin, a chemokine important in eosinophil migration and homeostasis (24–26), was measured in lung cell supernatants and lysates. If immune activation, combined with T supplementation, played a role in EMS susceptibility, then sensitized T-supplemented guinea pigs would be excepted to respond more vigorously to antigen challenge. In the guinea pig this would result in either increased levels of eosinophils in the tissues collected (especially lung or BAL) or a change in relative location of eosinophils (compared to sensitized animals not fed T), suggestive of differences in migration.

Materials and Methods

Animals and Diets. Forty-eight guinea pigs were obtained from Bio-Lab (St. Paul, MN). On arrival (Day 1) the guinea pigs weighed 200–250 g and were housed two per cage in approved animal research facilities. A standard vitamin C-fortified, pelleted diet (Teklad Guinea Pig Diet 7006 containing 178 g/kg protein, 2,640 kcal/kg energy, 27.9 g/kg fat, and 136.9 g/kg fiber; Harlan Teklad, Madison, WI) and fresh water were available *ad libitum*. Guinea pigs were orally supplemented for the last 14 days (Days 7–21) by gelatin capsule containing 200 mg (0.4 g/kg/day) of cellulose (C) or T. This dose of T approximates the median dose reported by EMS patients (27). The guinea pigs were weighed weekly and at the end of the experiment. Guinea pigs were euthanized by CO_2 asphyxiation immediately prior to tissue collection (Day 21). This protocol was approved by the Animal Care Committee of the University of Wisconsin.

Reagents and Solutions. L-Tryptophan (catalog no. T0254, lot 56H0421, produced in 1996, after Showa Denko KK stopped manufacture of L-tryptophan), ovalbumin (OA), collagenase (type I), Tris (8.5 pH), Triton X-100, bovine serum albumin (BSA), hematoxylin & eosin (H & E) stain, alkaline phosphatase conjugated goat anti-rabbit secondary antibody, disodium *p*-nitrophenyl phosphate (5 mg tablets), calcium ionophore$_{A23187}$ (CaI), fetal calf serum, RPMI 1640, gentamicin, amphotericin, penicillin/streptomycin, ethylenediaminetetraacetic acid (EDTA), non-nutritive cellulose (C), and components of buffers were all obtained from Sigma Chemical Co. (St. Louis, MO). Gelatin capsule (#3 in size) were obtained from Eli Lilly, Inc. (Indianapolis, IN).

The phosphate-buffered saline (PBS) used consisted of (m*M*) NaH_2PO_4, 1.9; Na_2HPO_4, 9.8; NaCl, 159. The Tyrode's physiological salt solution plus gelatin (TG) consisted of (m*M*) NaCl, 137; KCl, 2.6; NaH_2PO_4, 0.35; $NaHCO_3$, 11.9; glucose, 5.5; gelatin 1 g/l, adjusted to pH 7.4 with HCl. TGCM is TG with added $CaCl_2$ (2 m*M*) and $MgCl_2$ (1 m*M*).

Purified guinea pig eotaxin and rabbit anti-guinea pig eotaxin polyclonal antibody were generous gifts from Dr. Marc Rothenberg (Children's Hospital Medical Center, Cincinnati, OH).

Experimental Design. Two separate studies of 24 guinea pigs each (six treatments with four animals per treatment) were conducted. Each cage of two guinea pigs was assigned to one of the following six treatment groups (Table I): (i) unsensitized and cellulose-supplemented (UC); (ii) unsensitized and tryptophan-supplemented (UT); (iii) sensitized, sham (PBS)-challenged, and cellulose-supplemented (SSC); (iv) sensitized, sham (PBS)-challenged, and tryptophan-supplemented (SST); (v) sensitized, OA (antigen)-challenged, and cellulose-supplemented (SAC); (vi) sensitized, OA (antigen)-challenged, and tryptophan-supplemented (SAT). Logistically, four consecutive days of tissue processing were required, accordingly one guinea pig per treatment was started per day for four consecutive days, so that all guinea pigs received capsule supplementation for 14 days.

A schematic of the experimental design is shown in Fig. 1. Guinea pigs assigned to the sensitized treatments (i.e., SSC, SST, SAC, and SAT) were initially injected intraperitoneally (ip) with 0.05 mg of OA in $Al(OH)_3$ (20.4 g/ml) and PBS on Day 1. Guinea pigs assigned to the un-

Table I. Treatment Codes[a]

	Cellulose	Sigma L-tryptophan
Unsensitized	UC	UT
Sensitized/sham (PBS)-challenged	SSC	SST
Sensitized/antigen (OA)-challenged	SAC	SAT

[a] UC, unsensitized, cellulose; UT, unsensitized, tryptophan; SSC, sensitized/sham-challenged, cellulose; SST, sensitized/sham-challenged, tryptophan; SAC, antigen sensitized/antigen-challenged, cellulose; SAT, antigen-sensitized/antigen-challenged, tryptophan. Two studies conducted, n = 4/treatment, 6 treatments, 48 guinea pigs total.

sensitized treatments (i.e., UC and UT) were not injected. On Day 7, all guinea pigs were started on their assigned capsule supplementation of C or T. Guinea pigs on the sensitized treatments received subcutaneous (sc) flank injections (0.2 mg OA emulsified in Complete Freund's Adjuvant), and on Day 14 these guinea pigs received subcutaneous (sc) flank boost injections (0.2 mg OA emulsified in Incomplete Freund's Adjuvant). Eighteen hours prior to euthanization (Day 20), guinea pigs assigned to treatments SAC and SAT were challenged with intradermal (id) OA and PBS flank injections and guinea pigs assigned to treatments SSC and SST were sham-challenged with id (PBS) flank injections. This immune deviation sensitization protocol maximizes the production of IgG_1, the major reaginic antibody in the guinea pig (28). Eighteen hours prior to euthanization challenge was chosen because peak accumulaton of eosinophils following antigen challenge occurs between 12 and 24 hr in skin, lung, and BAL (29). In the second study, one group of guinea pigs exhibited a positive id reaction to the sham injection (possibly due to endotoxin contamination), therefore their results are not included in the data (n = 7 for treatments SSC, SST, SAC, and SAT).

Fluid and Tissue Evaluations. After the guinea pigs were euthanized by CO_2 asphyxiation, blood (collected from the jugular vein into 50-ml tubes containing 2 ml 2.7% EDTA in 0.85% NaCl, pH 7.2, for cell counts and red-topped Vacutainer® tubes for serum separation), bone marrow (left femur was removed and flushed with 20 ml of TG buffer), bronchial alveolar lavage fluid (BAL) (lungs removed and flushed through trachea with 20 ml of TG buffer), and lung cells (lungs digested with 125 U collagenase/g lung for 30 min, 37°C; monodispersed cells were resuspended to 20 ml) were collected. Total cell counts were done using a Coulter Counter (Model ZM, Coulter Corp., Miami, FL). Eosinophil counts were done manually using a hemacytometer by dilution in Unopettes® (Becton Dickinson, Rutherford, NJ) containing Phloxine B and counting stained eosinophils per manufacturer's directions. Eosinophil counts were expressed as total eosinophils × 10^6/ml recovered. Percent of total eosinophils recovered was also calculated for each tissue/fluid as follows:

$$\frac{\text{Eosinophils} \times 10^6/\text{ml recovered (blood, bone marrow, BAL, or lung)}}{\text{Sum of eosinophils} \times 10^6/\text{ml recovered from blood + bone marrow + BAL + lung}} \times 100$$

The percent of total eosinophils recovered is an indication of the relative distribution of the eosinophils between the different fluids and tissues evaluated at the time of sacrifice. Biopsies were taken from skin at the site of OA or sham challenge (as well as at unchallenged sites as controls), embedded in paraffin, sectioned, stained with H & E stain, and examined microscopically.

Eotaxin Direct ELISA. Enzyme-dispersed lung cells (10×10^6/ml) were incubated at 37°C for 24 hr with either TG buffer alone (spontaneous), CaI (positive control), or OA (antigen challenge). The cells were then pelleted, supernatants were collected, and cell pellets were lysed with 0.4% Triton X-100. Because only one anti-guinea pig eotaxin antibody could be obtained during the study, a direct ELISA approach was followed (30). Briefly, the cell supernatants and lysates, along with serial dilutions of purified guinea pig eotaxin (a standard curve from 0.125 to 32.0 μg/ml), were coated onto Immulon 2 HB plates (Dynex Technologies, Inc., Chantilly, VA) at a volume of 100 μg/

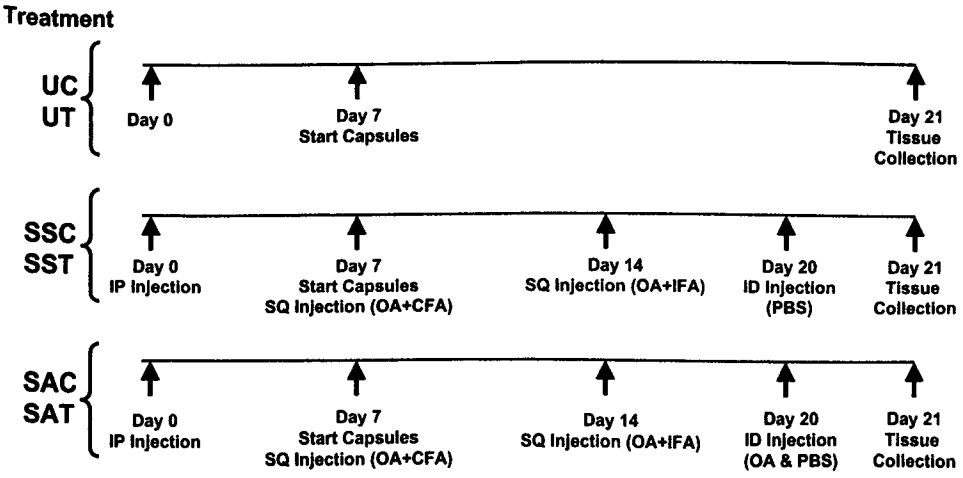

Figure 1. Schematic design of the experimental protocol. Abbreviations: IP = intraperitoneal; SQ = subcutaneous; OA = ovalbumin; CFA = Complete Freund's Adjuvant; IFA = Incomplete Freund's Adjuvant; ID = intradermal; PBS = phosphate buffered saline. (See Table I for treatment abbreviations.)

well, in duplicate and incubated (4°C, 24 hr). These coated plates were washed 3 times with washing buffer (0.1 *M* Tris, pH 8.5, 0.15 *M* NaCl) followed by addition of 100 μl/well of blocking buffer (washing buffer plus 2% BSA) and 45-min incubation at 37°C. The blocking solution was decanted, and 100 μg/well of a 1:2,000 dilution (in blocking buffer) rabbit anti-guinea pig eotaxin polyclonal antibody was added and incubated (room temperature, 2 hr). The plates were washed 3 times followed by the addition of 100 μl/well of a 1:100 dilution (in blocking buffer) of alkaline phosphatase conjugated goat anti-rabbit secondary antibody (room temperature, 2 hr). Next, the plates were washed 5 times followed by addition of 100 μl/well of disodium *p*-nitrophenyl phosphate in 50 m*M* Tris-HCl, pH 9.5 (final concentration 1 mg/ml, room temperature, 2 hr). The lower detection limit of the assay was 0.5 μg/ml. The absorbances were measured at 405 nm with a Bio-Tek EL_X808 automated microplate reader operated with KC4 KinetiCalc for Windows software (Bio-Tek Instruments, Inc., Winooski, VT), and the concentrations of eotaxin were calculated from the standard curve using a linear regression (KC4 KinetiCalc for Windows). The specificity of the polyclonal rabbit anti-guinea pig eotaxin antibody was previously demonstrated using immunohistochemistry (21).

Statistical Analysis. Results are presented as the mean ± SEM of eight animals. Data was analyzed using SAS® (SAS Institute, Cary, NC). A general linear-model, analysis of variance with preplanned comparisons was used to generate two-tailed *P* values. Fisher least-significant–difference tests were used to make appropriate post ANOVA comparisons. Preplanned comparisons included (i) treatment UC was compared with UT, treatment SSC was compared with SST, and treatment SAC was compared with SAT (these comparisons determined the effect of T supplementation compared to the animals sensitized the same, or unsensitized but without T); (ii) treatment UC was compared to each of the other five treatments (to see if the treatments had an effect compared to "control animals"); (iii) treatments on cellulose (UC, SSC, SAC) were compared to treatments on T (UT, SST, SAT) (to look for overall effect of T; comparisons were made only when there were no significant differences between the three C treatments and between the three T treatments). In order to equalize variance, eotaxin lysate data were square root transformed and percent eosinophil data were arc sine transformed prior to statistical analysis. A $P \leq 0.05$ was considered significant. However, since the small number of animals used per treatment (in an attempt at conserving animals) may have influenced significance, $P \leq 0.1$ will also be discussed.

Results

Growth. There were no differences in the weights of guinea pigs at the time of sacrifice for each of the six treatments. No obvious signs of discomfort were noted in any guinea pig, regardless of treatment.

Eosinophil Counts. The eosinophil counts obtained from the various tissues are shown in Fig. 2A–D. The SAT guinea pigs showed a significant decrease in blood eosinophils when compared to SAC guinea pigs [SAC (0.147 ± 0.021) $\times 10^6$ eosinophils/ml recovered vs SAT (0.086 ± 0.023) $\times 10^6$ eosinophils/ml recovered] and a significant increase in BAL eosinophils [SAC (0.033 ± 0.005) $\times 10^6$ eosinophils/ml recovered vs SAT (0.052 ± 0.008) $\times 10^6$ eosinophils/ml recovered]. No other significant differences were observed in eosinophil counts between C- and T-supplemented guinea pigs. Sensitization by itself resulted in an increase in lung eosinophils (UC, UT treatments versus SSC, SAC, SST, SAT treatments). Sensitization and OA challenge, but not PBS challenge, resulted in increased eosinophils in blood, BAL, and lung (19). The relative distribution of eosinophils between the different fluids and tissues is illustrated by the percent of total eosinophils recovered per fluid or tissue and is shown in Fig. 3A–D. Interestingly, the distribution of eosinophils in UT guinea pigs was more analogous to the sensitized guinea pigs (SSC, SST, SAC, SAT) than the UC guinea pigs. This difference in distribution was significant in bone marrow and lung (UC different from all other treatments, $P \leq 0.05$). That is, the relative locations of eosinophils in the UT animals were as if they were sensitized. (Eotaxin release results, below, confirm that these animals were not sensitized to OA, as their lung cells did not respond to OA challenge.) The total number of eosinophils recovered from the UT animals was not different from UC animals.

Eotaxin. As we have previously reported, unchallenged guinea pig lung cells spontaneously released eotaxin in vitro (21). The responses of guinea pig lung cells to CaI and OA challenge (with spontaneous release subtracted) are shown in Fig. 4A. Lung cells from UT guinea pigs tended to release more eotaxin in response to CaI challenge than UC guinea pigs. As they were not sensitized to OA, lung cells from UC and UT guinea pigs did not release detectable amounts of eotaxin in response to OA challenge. No differences were observed in the spontaneous release of eotaxin from lung cells, regardless of treatment (data not shown).

There were no differences between eotaxin levels measured in the lysates from the CaI and OA challenged cells (data not shown). However, significantly less eotaxin was found in lysates of unchallenged lung cells from T supplemented guinea pigs, compared to lysates of unchallenged lung cells from C supplemented guinea pigs (Fig. 4B, inset, $P < 0.05$). Mean values (ng/10^6 cells) were as follows: 0.0268 ± 0.008 (C) vs 0.006 ± 0.002 (T). Likewise, significantly less eotaxin was found in lysates of unchallenged lung cells from SAT guinea pigs, compared to lysates of unchallenged lung cells from UC guinea pigs.

Skin Biopsies. Tryptophan supplementation had no effect on the histopathology of the skin. Sensitization resulted in evidence of inflammation at the injection sites, as would be expected (31). The OA injection sites of the SA guinea pigs were erythematous and indurated, while the

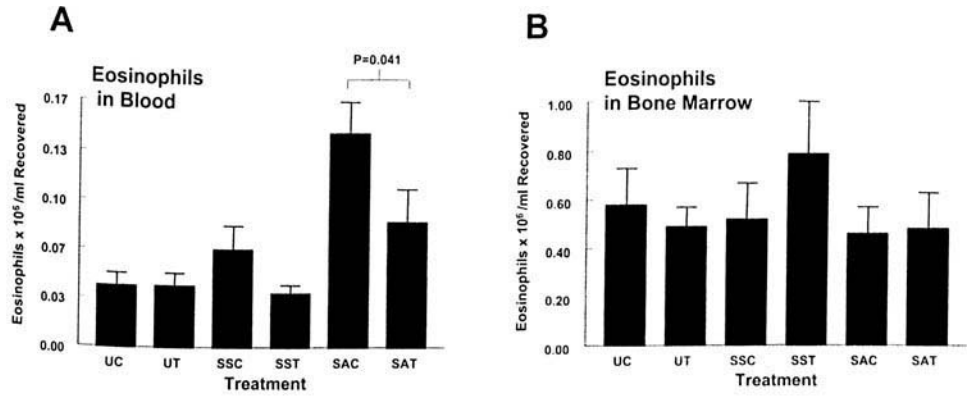

Figure 2. Eosinophil counts represent number of eosinophils × 10^6 per ml of fluid recovered from blood (A), bone marrow (B), and BAL (C), and eosinophils × 10^6 per ml of resuspended monodispersed lung cells recovered from the lung (D) (n = 8 or 7, see Methods).

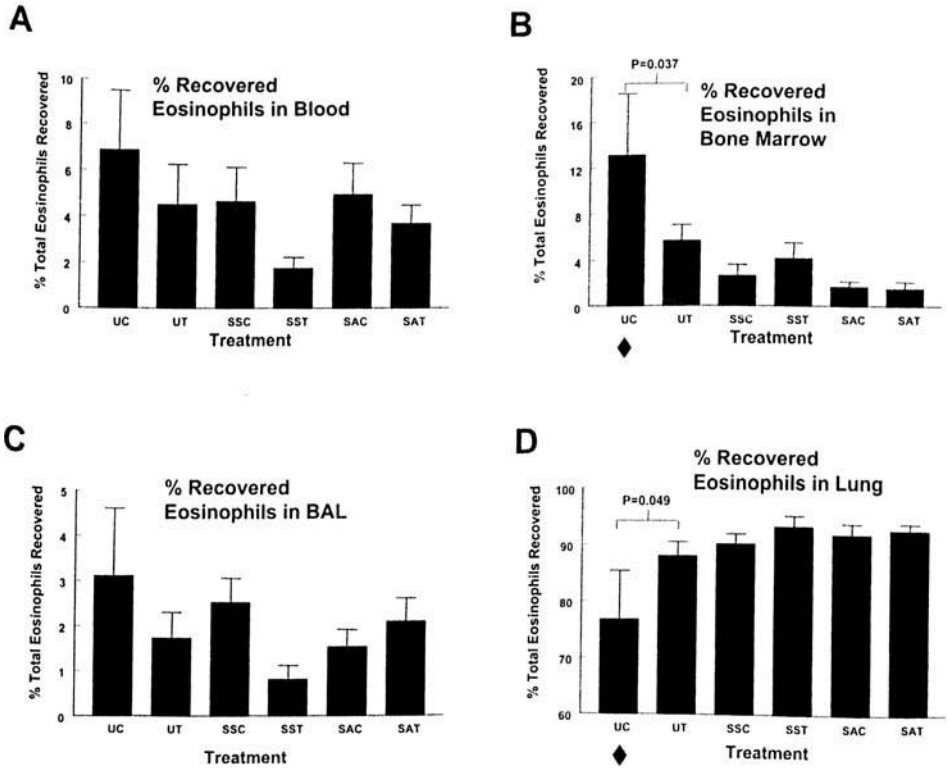

Figure 3. Percent total eosinophils recovered represents the relative distribution of total eosinophils recovered/guinea pig in blood (A), bone marrow (B), BAL (C), and lung (D) (n = 8 or 7, see Methods). The formula used to calculate this value was {[Eosinophils × 10^6/ml recovered (blood, BAL, bone marrow, or lung)]/(Sum of eosinophils × 10^6/ml recovered from blood + BAL + bone marrow + lung)} × 100. The diamond (♦) indicates that UC was significantly different from all other treatments in bone marrow and lung ($P \leq 0.05$).

sham (PBS) injection sites on both the SS and SA guinea pigs showed no macroscopic evidence of inflammation. Microscopic examination of the SA skin biopsies, as anticipated, showed a dramatic infiltration of inflammatory cells (eosinophil, neutrophils, mononuclear cells) in the dermis and subdermal tissue at the foci of inflammation. A great number of inflammatory cells were present even in the biopsies from SS (sham-challenged) guinea pigs.

Discussion

This is the first report, to our knowledge, of L-tryptophan, or any amino acid dietary supplement, having

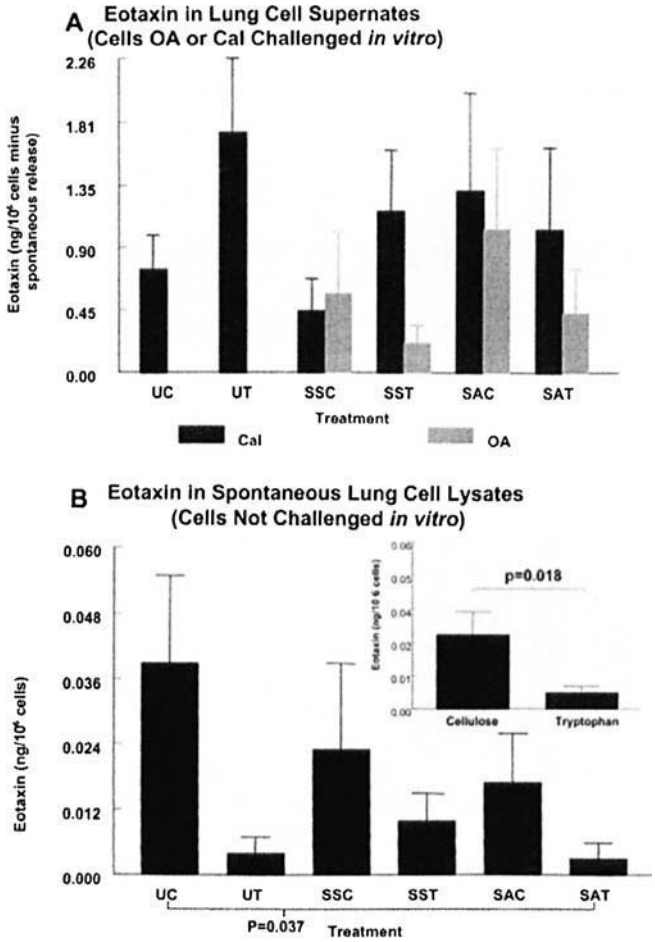

Figure 4. (A) Eotaxin concentrations measured in supernatants with spontaneous release subtracted (n = 8 or 7, see Methods), from monodispersed lung cells challenged for 24 hr with either calcium ionophore (Cal) (black bars) or ovalbumin (OA) (gray bars). Eotaxin was not detected in supernatants from monodispersed lung cells challenged for 24 hr with OA from either unsensitized, cellulose-supplemented animals (UC) or unsensitized, tryptophan-supplemented animals (UT). (B) Eotaxin concentrations measured in unchallenged monodispersed lung cells lysates (n = 8 or 7, see Methods) incubated for 24 hr. (Inset) Means of eotaxin concentrations in unchallenged monodispersed lung cells lysates from cellulose-supplemented animals (C) and tryptophan-supplemented animals (T) are significantly different ($P \leq 0.05$).

an effect on eosinophil and chemokine levels. It is important to note that we conducted preliminary studies with suspect Showa Denko T, as contaminants found in it are suspected to be at least part of the cause of EMS (6). In these preliminary studies, guinea pigs supplemented with suspect T at a dose of 0.4 g/kg/day responded very similarly to guinea pigs supplemented with the Sigma T used in the studies presented here (data not shown). These findings and the fact that Showa Denko no longer produces T led to use of only Sigma T in the current studies.

We chose to evaluate eosinophils in skin, bone marrow, peripheral blood, lung, and BAL. Our rationale for choosing these tissues was two-fold. First, the histopathologic findings in EMS involved the skin (perivascular, dermal, and fascial inflammation and fascial thickening with mononuclear cells with or without eosinophils) and the lung (interstitial, perivascular, and alveolar inflammation with mononuclear cells with or without eosinophils) (32). Second, an important part of our hypothesis involves changes in eosinophil migration in response to immune challenge, a process involving multiple organs. When a guinea pig is challenged with an antigen, eosinophils are recruited from the circulation and follow a chemokine gradient to the sight of challenge, in this case, the skin. In the guinea pig, the lungs also respond vigorously to antigen challenge regardless of the route of administration. Eosinophils are recruited from the circulation into the parenchymal tissues and finally through the bronchial epithelium into the alveoli where they can be recovered in the BAL. Eosinophils leaving the blood results in mobilization of eosinophils from the bone marrow into the circulation (33). Peak accumulation of eosinophils following antigen challenge occurs between 12 and 24 hr in skin, lung, and BAL (29), therefore we evaluated tissues collected in this time frame. If T ingestion resulted in irregularities in eosinophil migration, this might be reflected either by absolute differences in eosinophil counts in these tissues or by relative differences in the distribution of eosinophils between the tissues.

We observed an unanticipated decrease in peripheral blood eosinophils and an increase in BAL eosinophils in SAT guinea pigs, compared with SAC guinea pigs. This is likely due to enhanced migration of eosinophils from the peripheral blood to the bronchial alveoli.

We feel that the pattern of the relative distribution of eosinophils between the different fluids and tissues, summarized in Fig. 3A–D, is very important. It signifies that eosinophils in animals fed T but not sensitized (UT) are distributed similarly to eosinophils in sensitized guinea pigs (i.e., lesser percent recovered eosinophils in bone marrow, blood, and BAL and a greater percent recovered eosinophils in lung; UT was significantly different from UC in bone marrow and lung); that is, eosinophils in UT animals appear to migrate as if the animals were sensitized, and yet the lack of response of their lung cells to OA challenge (Fig. 4A) demonstrates that they are not sensitized to OA. This observation is even more intriguing when taken together with the decreased intracellular eotaxin in lung cells from tryptophan-supplemented animals even when unsensitized.

The kinetics of eotaxin generation and its relationship to eosinophil accumulation is an important feature of the guinea pig model for eosinophil migration. Humbles and co-workers studied these relationships in OA-sensitized guinea pigs that were subsequently challenged with aerosolized OA (29). They found that following aerosolized OA challenge, immunoreactive eotaxin levels in guinea pig airway tissue peaked at 6 hr and declined to low levels by 12 hr. Eosinophils appeared in the BAL 12–24 hr after challenge. Although we challenged with OA sc for a systemic response, our BAL eosinophil data agree with this time course. Unfortunately, direct comparison of eotaxin data between our studies is difficult as we looked at eotaxin

concentration on a per lung cell basis and Humbles and co-workers expressed it on a gram of lung basis.

Eotaxin, as previously mentioned, is important in regulation of eosinophil migration and homeostasis (24–26). Eotaxin is selective for eosinophils and can, in the presence of IL-5, stimulate rapid release of eosinophils from bone marrow resulting in blood eosinophilia (33). While many mediators have been recognized as eosinophil chemoattractants (such as leukotrienes, platelet activating factor, formyl methionyl leucylphenylalaine, and chemokines such as RANTES and MIP-1α), only eotaxin selectively promotes eosinophil recruitment (25).

We found less eotaxin in lung cell lysates from T-supplemented animals compared to C animals. This decreased level of eotaxin in the lung cell lysates of the T-supplemented guinea pigs could explain the findings of decreased blood and increased BAL eosinophils in the SAT compared to SAC animals. It suggests an increase in constitutive release of eotaxin in the T-supplemented guinea pigs (since eotaxin was decreased even in lung cell lysates from UT animals) and therefore could be an indication of alteration(s) in eotaxin regulation. In mice, eotaxin is a fundamental regulator of migration of eosinophils during normal, healthy states (26). We have previously shown that eotaxin message and protein are constitutively expressed in the airway epithelium of unsensitized guinea pigs (21). Therefore, this may be a significant finding, as it is believed that eotaxin is required for maintenance of baseline levels of tissue eosinophils (26) and might also explain the parallels between the distribution of eosinophils in the UT and sensitized guinea pigs.

Tryptophan metabolism could affect chemokine levels by several mechanisms. First, metabolism of T in guinea pigs, as in humans, involves IFNγ induction of the enzyme IDO to convert T to KYN through a pathway leading to QA, a neuroexitotoxin (34) and ultimately to NAD and NADP. While IFNγ has been shown to stimulate release of eotaxin synergistically along with IL-1β and TNFα (35), KYN and QA have also been shown to induce release of cytokines in vitro (36). As some EMS patients who were taking T were shown to have increased serum levels of IFNγ, KYN, and QA (17), it is reasonable to assume that these compounds could work in concert to result in immune activation with increased cytokine production and release. Furthermore, serum levels of IFNγ, KYN, and QA are increased in patients with various chronic diseases similar to those suffered by individuals who were taking T supplements, including autoimmune diseases, AIDS, sleep disorders, and depression (37, 38). It is possible that immune activation with increased IFNγ predisposes individuals to an altered tryptophan metabolism. Ingestion of large doses of T by these individuals could provide additional substrates to an already "primed" (up-regulated) enzymatic pathway. As previously mentioned, altered tryptophan metabolism has been described in EMS, eosinophilic fasciitis, and scleroderma (15). This "priming" could explain our results as well. Accordingly, it would be valuable in future studies to include evaluation of serum levels of IFNγ, IDO, KYN, and QA in guinea pigs as well as the direct effect of these compounds on guinea pig lung cells in vitro. While these studies cannot completely rule out the possibility that any amino acid supplementation could lead to hypereosinophilic disorders in susceptible individuals, the results obtained in this research warrant future study.

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