

Abbreviations used

cpm:	Counts per minute
DBPCFC:	Double-blind, placebo-controlled food challenge
IgE-MA:	IgE-mediated cow's milk allergy
ME:	Milk-induced enterocolitis syndrome
PBMCs:	Peripheral blood mononuclear cells
SI:	Stimulation index

proliferation assays should be used as a diagnostic or predictive test for food allergy.¹⁰⁻¹²

This study was designed to evaluate the clinical utility of *in vitro* lymphocyte proliferation assays in both IgE-mediated milk allergy (IgE-MA) and in milk-induced enterocolitis syndrome (ME). Many of the variables found in previous studies were controlled by using strict diagnostic criteria such as the double-blind, placebo-controlled oral food challenge (DBPCFC) for evaluation of our patient groups, by using autologous sera in the lymphocyte culture, by reporting and comparing our results in both cpm and SIs, and by analyzing statistical significance with nonparametric tests because the data are not normally distributed.

METHODS

Subjects

Patients were selected from the Pediatric Allergy Clinic and from children referred to us for evaluation of food allergy. The first patient group consisted of 27 children with IgE-MA confirmed by positive skin prick test responses, and inpatient DBPCFCs were performed as previously described.¹⁴ These children had immediate symptoms of allergy after milk ingestion, such as morbilliform rash, urticaria, pruritus, angioedema, vomiting, laryngeal edema, or wheezing. The median age of the children was 4 years. The second patient group consisted of nine children with ME diagnosed by the oral challenge procedure described by Powell¹⁵ or by a history of repeated episodes of isolated delayed vomiting more than 2 hours after the ingestion of milk protein. Patients with ME are not typically atopic and have no detectable IgE antibodies to milk; that is, they have negative skin test and RAST responses. Symptoms generally consist of vomiting, diarrhea, and colic; however, a small but significant percentage will experience hypotension and will require intravenous fluid resuscitation. They will display increased numbers of polymorphonuclear neutrophils on their differential white blood cell counts 4 to 6 hours after milk ingestion. Symptoms resolve completely within 48 to 72 hours of eliminating milk protein from the diet. Their symptoms recur once the milk-based formula is resumed. Up to 60% of these patients will also have similar reactions to soy antigen, if they begin receiving a soy-based formula at an early age.¹⁶ This association is less common in IgE-MA, in which concurrent allergy to soy antigens is seen in approximately 5% to 15% of children allergic to cow's milk (unpublished data). All the patients with ME had negative skin prick test responses to milk; the median age of this group was 1.3 years.

The control group comprised 21 children (median age, 5 years) who had no evidence of milk allergy. Sixteen of these children, although not allergic to milk, had other documented food allergies.

Lymphocyte culture

Before oral challenges, each patient had heparinized venous blood drawn in a sterile manner. All patients with milk sensitivity were on strict milk elimination diets before challenge. Peripheral blood mononuclear cells (PBMCs) were purified by centrifugation on a Ficoll-Hypaque gradient, washed twice, and resuspended in RPMI-1640 (GIBCO Laboratories, Grand Island, NY) containing L-glutamine (2 mmol/L), penicillin (100 U/ml), streptomycin (100 µg/ml), and 5% autologous serum. Preliminary studies indicated that optimal proliferative responses were obtained with autologous sera compared with pooled human AB sera or fetal calf serum. Cells were plated at 2×10^5 cells/200 µl/well in round-bottom microtiter plates.

Stock solutions of food antigens were prepared in RPMI-1640. The final concentrations in culture were as follows: milk antigens were a mixture of casein at 1000 µg/ml and whey at 100 µg/ml or a mixture of casein at 500 µg/ml and whey at 50 µg/ml; soy protein concentrations were 250 µg/ml and 125 µg/ml. Milk was chosen as the food antigen of interest, and soy antigen was chosen as a nonrelevant food antigen. The food antigen concentrations were chosen for optimal proliferation as described by Van Sickle et al.⁸ Similarly, a recent study, in which 7 logarithmic dilutions of milk proteins (10^{-2} to 10^{-8} mg/ml) were used, demonstrated optimal proliferation with the highest concentrations of milk protein.¹³ Casein and whey proteins were kindly provided by Ross Laboratories (Columbus, Ohio). Soy antigen was prepared by dissolving soy flour (Arrowhead Mills, Hereford, Texas) in phosphate-buffered saline and solubilizing the soy protein. All food antigens were sterile-filtered (Millipore, Bedford, Mass.) and frozen at -20°C until use.

Tetanus toxoid (lot no. 40106-515; Massachusetts Department of Public Health) was used as a positive control antigen to which all immunized children should have a cellular response. The final concentration per well for tetanus antigen was 0.2 Lf/ml.

Culture with each antigen concentration was performed in quadruplicate. Negative control cultures (no antigen added) were prepared simultaneously. Cultures were incubated at 37°C in a 5% CO_2 environment. Cells were pulsed for 4 hours with 1 µCi of tritium-labeled thymidine per well on days 5, 7, and 9. Cells were harvested on glass fiber filter paper by using a cell harvester (Cambridge Technologies, Inc., Cambridge, Mass.). Incorporated cellular radioactivity was measured with a liquid scintillation counter (Beckman Instruments, Inc., Irvine, Calif.) with toluene and 2,5 diphenyloxazole.

Statistical analysis

Mean cpm were calculated for each antigen concentration and for background control wells. Results were expressed in cpm and as the SI, which is the mean cpm for antigen-stimulated cells divided by the mean cpm for unstimulated cells. The cpm or SI reported is the maximal value for each antigen on day 5, 7, or 9. In most patients, maximal proliferation occurred on day 7 with the higher antigen concentration.

Comparative analyses of the results were done by using the Wilcoxon rank-sum test because the data were not normally distributed.

RESULTS

The median proliferative response, in cpm, to milk antigen in the 27 children with IgE-MA was significantly greater than that in the control patients ($p = 0.001$) (Fig. 1). Although the difference between medians in these two groups was significant, the ranges were quite large

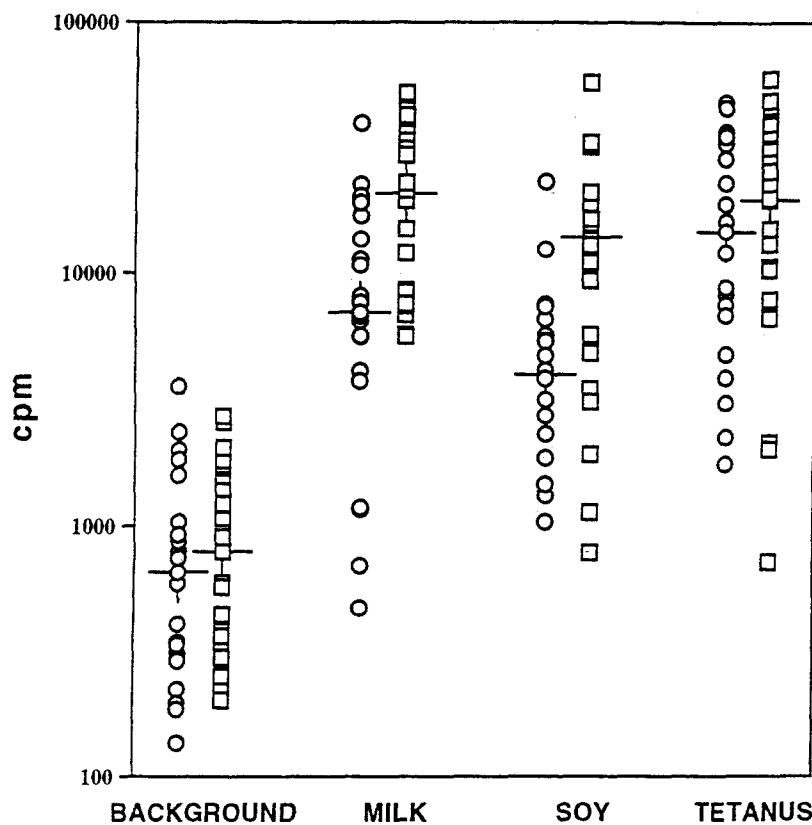


FIG. 1. Comparison (in cpm) of lymphocyte proliferation in response to antigen stimulation in control subjects (○) and in patients with IgE-MA (□).

and overlapping. There was no significant difference between the patients with IgE-MA and the control subjects in background proliferation or in cellular response to tetanus antigen. When these two groups were compared for their response to soy, eight of the children with milk allergy and three of the control subjects were not used in this comparison because they had documented soy antigen allergy; therefore, soy antigen was not a nonrelevant food antigen in these 11 patients. Interestingly, the 19 children with milk allergy who were not allergic to soy antigen had a significantly greater response to soy antigen (median, 13,058 cpm) than did the 18 control patients who were not allergic to either soy or milk antigens (median, 4,427 cpm; $0.01 < p < 0.05$). As seen in Fig. 1, there was also a large overlap between the two groups in their cellular response to soy antigen.

When the patients with IgE-MA were compared with control patients by using SIs instead of cpm (Fig. 2), the statistical significance was less striking. The median SI in response to milk antigen in the group with milk allergy was 19.2, compared with 14.2 in the control subjects ($p < 0.05$). Again, the overlap of responses was quite large. When the SIs of these two groups were compared for their response to soy antigen (excluding the patients with

soy allergy in both groups, as before), there was no significant difference in median SI values ($p > 0.10$). There was no significant difference between any of the groups when their responses to tetanus were compared by using SIs or cpm.

The cellular proliferation in response to milk was compared in the second patient group, the nine children with ME, and the 21 control patients (Fig. 3). The median cpm in the patient group was 11,975 cpm, a value that was significantly higher than the median value found in the control subjects (6,969 cpm; $0.01 < p < 0.05$). Background proliferation and response to tetanus were not significantly different between the two groups. Proliferation in response to soy as a nonrelevant antigen was not used in this patient group because the majority of the patients with ME had either documented reactions to soy antigen or had never ingested soy antigen before their enrollment in the study. When the proliferative response to milk antigen was compared in the patients with ME and the control subjects by using SIs instead of cpm (Fig. 4), the difference was not statistically significant ($p < 0.2$).

When the lymphocyte responses of patients with ME were compared with those of the patients with IgE-MA, there was no significant difference in their response to

milk antigen ($p = 0.812$) or tetanus toxoid or in their background proliferation. There was also no significant difference when SIs were used instead of cpm.

Because most patients and control subjects demonstrated significant proliferative responses to milk antigens, one might question whether milk proteins have mitogenic properties. Although the optimal response to milk proteins is at 7 days, suggesting an antigenic response, we stimulated six egg antigen-specific T-cell lines with milk antigens to rule out any mitogenic response to these proteins. Antigen-specific T-cell lines were used to rule out a mitogenic response to milk antigens because even cord blood lymphocytes have been shown to respond to food antigens, as a result of in utero exposure and sensitization.¹⁷ The egg antigen-specific T-cell lines demonstrated no proliferation above background to milk antigens but had significant proliferation to egg proteins (median counts: background, 4970 cpm; milk antigen, 4473 cpm [SI, 0.9]; and egg antigen, 11,928 cpm [SI, 2.4]), suggesting no mitogenic response to the milk proteins.

DISCUSSION

We have evaluated and compared the lymphocyte proliferative responses to milk antigen in 27 children with IgE-MA, nine children with ME, and 21 children without milk allergy. When compared as groups with the control group, both the patients with IgE-MA and the patients with ME showed significantly increased proliferative responses to milk antigen. However, the ranges of all three groups' responses were quite large and overlapped extensively when either cpm or SIs were compared. Many control patients demonstrated in vitro proliferative responses to milk that were higher than those of some patients with milk allergy. Interestingly, patients with IgE-MA had higher proliferative responses to soy antigen than did the control patients. Because none of the patients in this particular comparison were allergic to soy antigen, it would suggest a lack of specificity in food antigen-stimulated lymphocyte responsiveness or that patients with food allergy have a greater cellular reactivity to a variety of food antigens. There were no differences among the groups in overall lymphocyte responsiveness to a common recall antigen, as reflected by the proliferation in tetanus antigen-stimulated cultures or by activation status, as seen in the unstimulated cultures.

Our results are in agreement with those of an early study by May and Alberto,⁵ who found significant lymphocyte stimulation in response to food proteins in children with or without allergy. Scheinman et al.⁷ reported positive lymphoblast transformation to α -lactalbumin or β -lactoglobulin in 17 of 38 patients with "cow's milk protein intestinal intolerance." They found only four patients of 43 to be positive in their nonatopic control group. A more recent study claimed that the lymphocyte stimulation test was specific, despite results that showed a large overlap in lymphocyte proliferation in response to β -lactoglobulin in both patient and con-

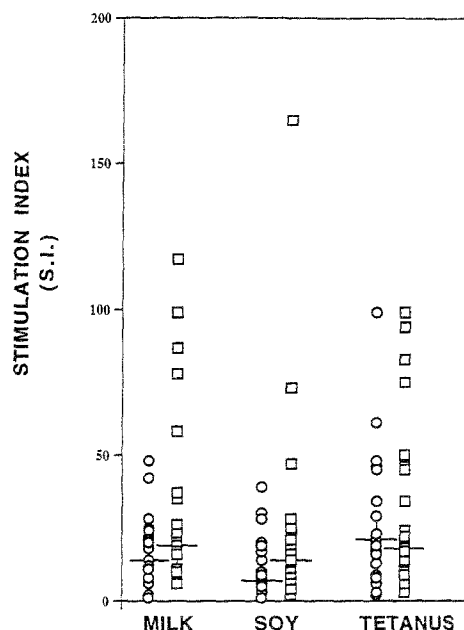


FIG. 2. Comparison (in SIs) of control subjects (○) and patients with IgE-MA (□).

trol groups.¹⁰ In that study, open oral challenges were used to confirm milk allergy in the patient group; some patients reportedly reacted up to 24 hours after ingestion of milk.

In a recent study by Suomalainen et al.,¹⁸ lymphocyte responses to cow's milk proteins were reported to decrease if blood from patients with milk allergy was drawn after a positive milk challenge. (In our study, all blood samples were drawn before food challenges.) In their study, milk challenges were performed openly over a 48-hour period; whole blood was used in lymphocyte cultures, and iodine 125-labeled 5-iodo-2' deoxyuridine was added 16 to 18 hours before cell harvest on day 7. Despite these methodological differences, results of this study were similar to those reported here, showing large overlaps in lymphocyte proliferative responses to milk in both patients and control subjects. Suomalainen et al.¹⁸ also observed a difference in statistical significance when results were analyzed by using cpm rather than SIs.

In another study, Tainio and Savilahti⁹ reported a sensitivity of 88% and a specificity of 67% when a combination of lymphocyte proliferative responses to β -lactoglobulin and specific IgE antibodies to cow milk was measured. Patients were diagnosed with milk allergy on the basis of an open oral milk challenge protocol in which the ingested quantity of milk protein was increased every 4 hours, up to 72 hours, until a reaction was observed. In this study, patients with milk allergy were diagnosed on the basis of DBPCFCs, as described previously.¹⁴ Another difference included performing the thymidine pulses on the third day of the culture. Although frequently optimal for mitogen-stimulated lymphocytes, 3 days of culture is generally too short a

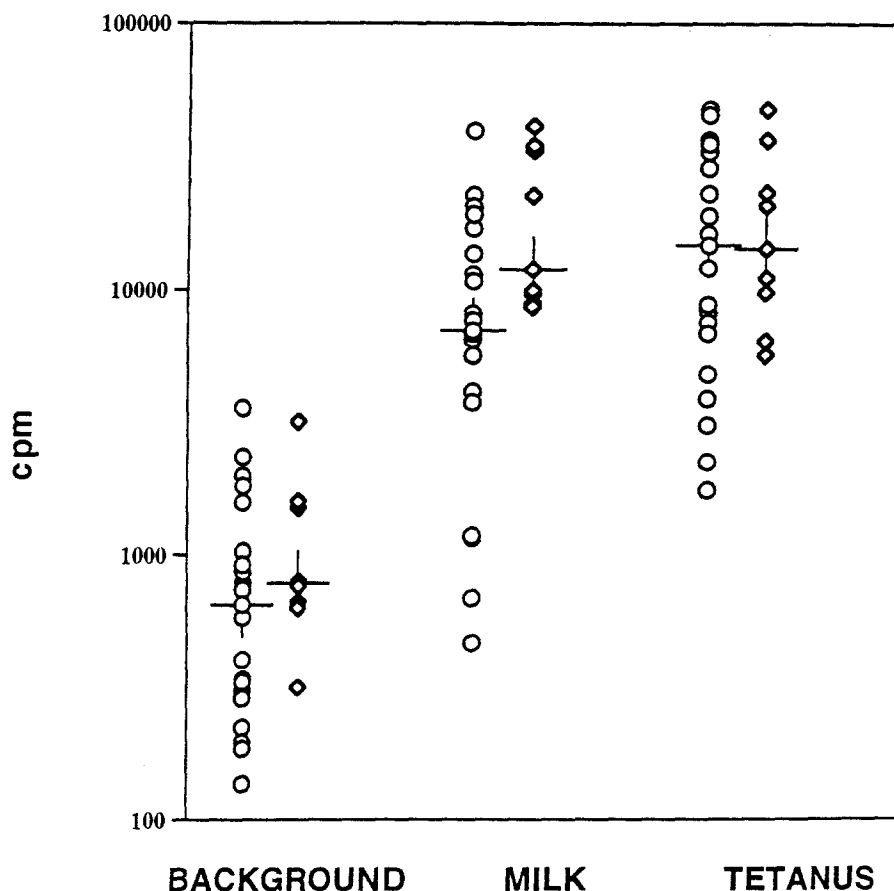


FIG. 3. Comparison (in cpm) of control subjects (○) and patients with ME (◇).

time in which to achieve peak proliferation caused by food protein stimulation, which requires antigen presentation. In this study peak proliferation generally occurred on day 7, which is in agreement with results of previous studies.^{5, 8, 10, 18}

Findings in this study differ from those of an earlier study of patients with atopic dermatitis by Kondo et al.¹¹ On the basis of food challenges, patients were divided into those with immediate symptoms and those with late (>2 hours) symptoms of allergy to food. In contrast, our patients with IgE-MA, 21 of whom had atopic dermatitis (AD), all had positive DBPCFCs, with symptoms manifesting within 2 hours of ingestion of milk, consistent with previous studies^{14, 19, 20} that used the challenge protocol initially described by May.²¹ Another methodological difference in the study by Kondo et al.¹¹ was the use of bovine serum albumin as the stimulating milk protein, whereas casein and whey were used in this study. Kondo et al.¹¹ reported peak proliferation on days 3 through 5, in contrast to day 7 in our study. Their mean cpm and SI values were quite low in their group with milk allergy (<2000 cpm; SI, <4, respectively) with wide overlapping ranges. Our median cpm and SI values were much greater (>2000 cpm; SI, >19), although also with wide overlapping ranges. Although the study by Kondo

et al.¹¹ states that the results of patient and control groups do not reach statistically significant differences, they still suggest that "the combination of RAST and the detection of proliferative responses of PBMCs to each food antigen is very useful in the diagnosis of hypersensitivity in children with AD who are sensitive to food allergens."¹¹ The results of our study and those of others do not support the contention that lymphocyte proliferation assays are useful in the diagnosis of food allergy. However, in the rare cases in which lymphocyte proliferation to milk proteins *in vitro* is absent, it is very unlikely that a patient would experience an allergic response because some lymphocyte activation is necessary for both hypersensitivity and normal immune responses to food antigens.

Patients with ME in this study had PBMC proliferation in response to milk antigen similar to that reported by Van Sickle et al.⁸ However, our control group had a wider range of proliferative responses to food antigens and a higher median proliferation than those seen in the study by Van Sickle et al.,⁸ in which the control group's proliferative responses did not overlap with the responses of the patient group. This difference might be explained by the young age of the patients in their control group (mean age, 4.7 months). Also, none of the

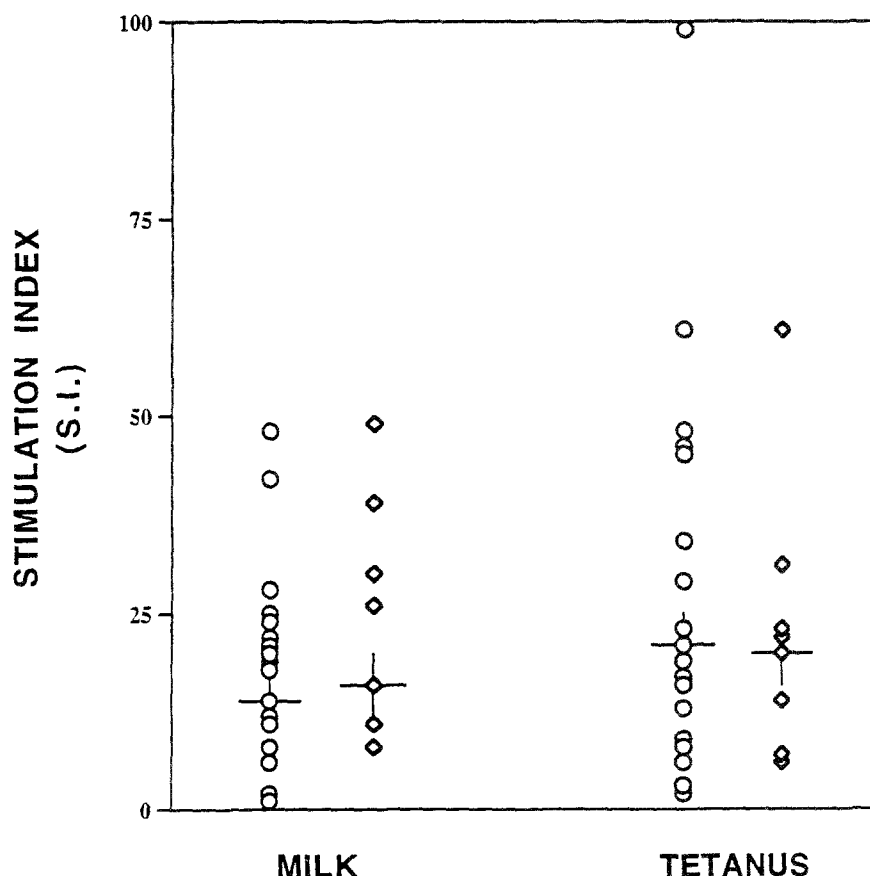


FIG. 4. Comparison (in SIs) of control subjects (○) and patients with ME (◇).

patients in their control group were known to be atopic. Although not statistically significant, patients with IgE-MA in our study tended to have higher proliferative responses to milk than did the patients with ME; the latter group is affected by a non-IgE-mediated disorder in which cell-mediated hypersensitivity is believed to play a prominent role. Although the higher proliferative responses overall in both patient groups might implicate a pathogenic role for sensitized lymphocytes in milk allergy and in ME, the wide range of responses in both patients and control subjects would suggest that proliferation alone does not sufficiently reflect the immunologic mechanisms involved in the clinical manifestations of these conditions.

In conclusion, the lymphocyte proliferation assay does not appear to be useful as a diagnostic test in food hypersensitivity. Significant lymphocyte proliferation occurs in patients with IgE-MA, in patients with ME, and in control subjects. Future investigation into the profile of cytokines elaborated by lymphocytes proliferating in response to food antigen stimulation may provide insight into the role of cell-mediated immunity in the pathogenesis of food hypersensitivity.

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The relationship between airway hyperreactivity (AHR) and sodium, potassium adenosine triphosphatase (Na^+, K^+ ATPase) enzyme inhibition

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Previous studies have documented the presence of a sodium, potassium adenosine triphosphatase (Na^+, K^+ ATPase) enzyme inhibitor on platelet membranes and in the plasma of patients with allergy, many of whom historically had airway hyperreactivity (AHR). The purpose of this study was to investigate the relationship between methacholine AHR and Na^+, K^+ ATPase enzyme inhibition. In the first experiment, 47 adult subjects (13 allergic, 5 potentially allergic, 12 asthmatic, and 17 control subjects) were tested for platelet membrane Na^+, K^+ ATPase inhibition and AHR. Area under the methacholine dose-response curve (AUC) was expressed as percent baseline $\text{FEV}_1 \times \log$ concentration of methacholine ($\log [\text{mg/ml}]$) and plotted as a function of the difference in postfreezing and prefreezing platelet membrane Na^+, K^+ ATPase activities (reflective of membrane-bound inhibitor), which was expressed as nanomoles per microgram of protein per minute ($\text{nmol}/\mu\text{g}$ protein/min). A significant ($r = -0.44, p < 0.005$) negative correlation between the two was detected, such that high levels of AHR (low AUC) were associated with high levels of membrane-bound inhibitor. To test for a causal relationship between the two, the ability of a Na^+, K^+ ATPase inhibitor to directly influence the level of AHR was determined in a second experiment. Eight allergic and 10 control subjects were administered AHR tests on 2 different days, immediately after inhalation of either nebulized ouabain (1 mg) or placebo in a double-blind fashion. Ouabain versus placebo inhalation decreased the PC_{20} in four of the patients with allergy. Additionally, ouabain increased methacholine AHR in patients with allergy, as manifested by a lower AUC in seven of the eight patients. In contrast, the mean AUC for the ouabain versus placebo prechallenges did not change significantly in the control group. Finally, a positive correlation was demonstrated between the levels of platelet membrane Na^+, K^+ ATPase inhibition and bronchial responsiveness to ouabain ($r = 0.49, p < 0.05$). These results provide both correlative and mechanistic evidence for a causal relationship between Na^+, K^+ ATPase enzyme inhibition and AHR. (*J Allergy Clin Immunol* 1997;99:367-73.)

Key words: Sodium, potassium adenosine triphosphatase, ouabain, methacholine, airway hyperreactivity, allergy, asthma

The biochemical defects responsible for allergic diseases, including asthma, are unknown. However, a de-

Abbreviations used

AHR:	Airway hyperreactivity
AUC:	Area under the methacholine dose-response curve
MDI:	Metered-dose inhaler
Na^+, K^+ ATPase:	Sodium potassium adenosine triphosphatase enzyme

fect involving Na^+ metabolism has been postulated.^{1,2} Recent epidemiologic studies have shown an increase in bronchial reactivity with increasing dietary salt intake, thereby suggesting that cell membrane Na^+ transport systems may be important in determining airway smooth muscle contractility and bronchial reactivity in human beings.²⁻⁵ Indeed, other studies have suggested a role for the sodium potassium adenosine triphosphatase enzyme (Na^+, K^+ ATPase) in determining airway smooth muscle contractility.^{1,6-10} For example, ouabain, a potent Na^+, K^+ ATPase inhibitor, has been shown to: (1) contract human and guinea pig airway smooth muscle in vitro^{1,7,8} and (2) increase histamine responsiveness in guinea pigs in vivo.⁹

More recently, our laboratory has documented a reduction of Na^+, K^+ ATPase activity in freshly isolated platelet membranes of patients with allergy, many of whom had airway hyperreactivity (AHR).¹⁰⁻¹² A follow-up study showed that the reduction in Na^+, K^+ ATPase activity was due to an enzyme inhibitor that was displaced from the platelet membrane by the freezing process. Moreover, a soluble Na^+, K^+ ATPase inhibitor with an extracellular binding site was also detected in the plasma of these same subjects.^{11,13} Whether Na^+, K^+ ATPase is regulated by an endogenous inhibitor in vivo is unclear. Moreover, the possible role of an Na^+, K^+ ATPase inhibitor in the pathogenesis of AHR is not known. However, the potential consequences of a reduction in Na^+, K^+ ATPase activity include an alteration of electrical membrane potential,¹⁴ changes in the levels of intracellular cations,¹⁵ and downregulation of responses initiated by binding of β -agonists to the β -adrenergic receptor.¹⁶ Each of these mechanisms is potentially involved in changing the level of responsiveness of cells involved in the pathogenesis of allergic diseases, including AHR.^{17,18}

The purpose of these experiments was to investigate

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the role of Na^+, K^+ ATPase inhibition in human AHR. The first experiment was designed to determine whether a correlative relationship exists between the two parameters. The level of Na^+, K^+ ATPase inhibition was assessed by using the blood platelet, a circulating inflammatory cell, which has been used in previous experiments. The second experiment was designed to determine whether experimental treatment with an Na^+, K^+ ATPase inhibitor results in modulation of the level of AHR in vivo.

METHODS

Approval for both experiments was given by the Human Rights Committee of Children's Hospital of Pittsburgh, and informed written consent was obtained from all subjects.

Exclusion criteria for both experiments included: (1) symptoms of allergic, asthmatic, or infectious conditions at the time of study; (2) a respiratory tract infection within the past 6 weeks; (3) the presence of hypertension or other chronic diseases as determined by history and physical examination; and (4) a baseline FEV_1 of less than 80% of predicted value.

Experiment 1

Subjects. Four groups of adult subjects were identified on the basis of history and results of physical examination and laboratory studies. The first group ($n = 13$) consisted of subjects with allergic rhinitis only, defined by (1) the presence of seasonal and/or perennial nasal symptoms, results of a physical examination consistent with allergic rhinitis, and positive intradermal skin test responses (>5 mm wheal) to pollens, molds, or dust mites; and (2) the absence of a diagnosis of asthma. The second group ($n = 12$) consisted of subjects with allergic rhinitis and asthma, defined by (1) the presence of allergic rhinitis as described above and (2) a documented diagnosis of asthma.¹⁹ The third group ($n = 5$) consisted of subjects with the potential for allergic rhinitis, defined by (1) the absence of seasonal and perennial nasal symptoms and negative results of physical examination but the presence of positive intradermal skin test responses to pollens, molds, or dust mites; and (2) the absence of a diagnosis of asthma. The fourth group ($n = 17$) consisted of control subjects, defined by (1) the absence of seasonal and perennial nasal symptoms, negative results of physical examination, and negative intradermal skin test responses to pollens, molds, and dust mites; and (2) the absence of a diagnosis of asthma. The age range of the 47 subjects was 19 to 45 years (mean age, 28 years). Twenty-three of the subjects were female, and 24 were male.

Of the subjects with asthma, two were using theophylline prophylactically, seven had previously used β -adrenoceptor agonists administered by metered-dose inhalers (MDIs) on an as-needed basis, and none had used corticosteroids for at least 6 weeks. Except for daily thyroid hormone replacement therapy in one normal and one potentially allergic subject who had histories of hypothyroidism, none of the other subjects had used any medications within the past 6 weeks. Only one potentially allergic subject was a regular cigarette smoker.

Protocol. On the study day, a 10 ml venous blood sample was obtained for assay of prefreezing and postfreezing platelet Na^+, K^+ ATPase activity. Intradermal skin tests (100 protein nitrogen units/ml) with 14 different pollens, molds, and dust mites were applied; and methacholine bronchial provocation testing was performed as previously described.²⁰

Platelet preparation and Na^+, K^+ ATPase assay. Platelets were isolated, sonicated, and serially centrifuged; and the resultant particulate fraction was resuspended in 1 ml of Tris-sucrose as previously described.¹⁰⁻¹² A portion of the particulate fraction was assayed in duplicate for Na^+, K^+ ATPase activity within 2 hours of sample collection. The remainder of the platelet particulate fraction was frozen immediately at -70°C , thawed 1 to 4 weeks later, and assayed for Na^+, K^+ ATPase activity.

The prefreezing and postfreezing platelet particulate fractions were assayed for Na^+, K^+ ATPase activity at 37°C by the linked pyruvate kinase-lactate dehydrogenase spectrophotometric method. The reaction mixtures for these assays and the method for calculating Na^+, K^+ ATPase activity have been described previously.¹⁰⁻¹³ Protein content was assayed by the Lowry method, and enzyme activities were expressed as nanomoles per microgram of protein per minute ($\text{nmol}/\mu\text{g protein}/\text{min}$).²¹ The coefficient of variation was 10% for a single prefreezing sample assayed 11 times for Na^+, K^+ ATPase (intraassay), 11% for a single postfreezing sample assayed 10 times in the same Na^+, K^+ ATPase assay (intraassay), and 7% for a single postfreezing sample assayed 10 times in 10 different Na^+, K^+ ATPase assays (interassay).

Methacholine challenge. After baseline measurements of FEV_1 were obtained, the challenge was performed by administering ascending serial concentrations of methacholine (Roche Laboratories, Nutley, N.J.). Five dosimeter-controlled inhalations of each methacholine concentration (0, 0.025, 0.25, 2.5, 10, and 25 mg/ml) were delivered by nebulization, and duplicate measurements of FEV_1 were obtained within 5 minutes of each administration. The challenge was ended when there was a 20% or greater reduction in FEV_1 compared with the baseline value or when the 25 mg/ml dose had been administered.²⁰

Data analysis. Methacholine dose-response curves were constructed for each subject by plotting the FEV_1 obtained at baseline and after the administration of each methacholine dose (expressed as percent baseline FEV_1) against the log of the methacholine concentration ($\log [\text{mg}/\text{ml}]$). Areas under the methacholine dose-response curve (AUCs) were calculated for each subject ($\text{percent baseline } \text{FEV}_1 \times \log [\text{mg}/\text{ml}]$) and plotted as a function of platelet Na^+, K^+ ATPase inhibition ($\text{postfreezing} - \text{prefreezing } \text{Na}^+, \text{K}^+ \text{ ATPase } [\text{nmol}/\mu\text{g protein}/\text{min}]$). This method of analysis allows for comparisons across the entire methacholine dose-response curve and has been previously published.²² A linear regression was then performed between the two parameters.²³

Experiment 2

Subjects. In this experiment, two groups of subjects were identified: (1) 10 subjects with allergic rhinitis, defined as described above for Experiment 1 (4 subjects in this group also had a diagnosis of asthma); and (2) 10 nonallergic control subjects, defined as described above for Experiment 1 (group 4). All of the subjects in this experiment had participated in the first experiment. The age range of the 20 subjects was 19 to 30 years (mean age, 26 years). Ten of the subjects were female, and ten were male.

Of the asthmatic subjects, one was using theophylline prophylactically, and two had previously used a β -adrenoceptor agonist MDI as needed. With the exception of one subject who was using naproxen, none of the control subjects had used any medications for 6 weeks before the experiment. None of the subjects in this experiment were regular cigarette smokers.

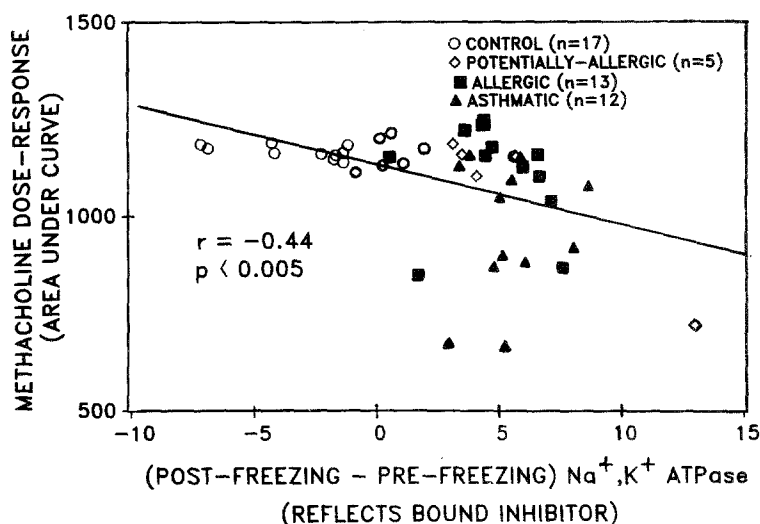


FIG. 1. Correlation between platelet Na^+, K^+ ATPase inhibition (postfreezing – prefreezing Na^+, K^+ ATPase expressed as nanomoles per microgram of protein per minute) and bronchial methacholine responsiveness (AUC expressed as percent baseline $\text{FEV}_1 \times \log [\text{mg/ml}]$) in 47 subjects.

Protocol. On the study day, a venous blood sample was obtained for assay of prefreezing and postfreezing platelet membrane Na^+, K^+ ATPase activity as described above. Intradermal skin tests (100 protein nitrogen units/ml) with 14 different pollens, molds, and dust mites were applied.

On 2 different days, separated by at least 5 days, either 1 mg of nebulized ouabain (Sigma Chemical Co.) or placebo (deionized water) was administered by oral inhalation over a 10-minute period in a random, double-blind fashion after baseline measurements of FEV_1 were obtained. Ten minutes after the ouabain or placebo inhalation, a methacholine bronchial provocation test was administered.²⁰ The methacholine challenge was not administered if there was a 20% or greater decrease in FEV_1 after ouabain or placebo inhalation. Blood pressures and pulses were monitored during the ouabain or placebo inhalations, and no effects on either parameter were noted.

Data analysis. Methacholine AHR was expressed as PC_{20} and AUC (percent baseline $\text{FEV}_1 \times \log [\text{mg/ml}]$), which was calculated as described in Experiment 1.²² For each group, the values obtained after ouabain versus placebo inhalation were compared, and the level of statistical significance was determined by the paired *t* test.²⁴ A linear regression was performed between platelet membrane Na^+, K^+ ATPase inhibition (postfreezing – prefreezing Na^+, K^+ ATPase [nmol/ μg protein/min]) and bronchial responsiveness to ouabain.²³ The bronchial responsiveness to ouabain was determined by calculating the difference between methacholine AHR (AUC expressed as percent baseline $\text{FEV}_1 \times \log [\text{mg/ml}]$) after placebo and ouabain inhalation.

RESULTS

In the first experiment, the relationship between methacholine AHR and Na^+, K^+ ATPase inhibition was examined for a possible correlation. For each subject, an AUC was calculated and plotted as a function of the difference between postfreezing and prefreezing platelet membrane Na^+, K^+ ATPase activities (Fig. 1). Previous work has shown that the freezing-associated increase in platelet Na^+, K^+ ATPase activity correlated with the

amount of Na^+, K^+ ATPase inhibitory activity released from the platelet membranes during the freezing process. In this study, the subjects with allergic diseases (allergic rhinitis and asthma) and potentially allergic diseases displayed large increases in platelet membrane Na^+, K^+ ATPase activity after freezing as compared with the control subjects. The mean ($\pm$ SD) respective prefreezing and postfreezing Na^+, K^+ ATPase activities were 4.33 ± 2.07 nmol/ μg protein/min and 2.69 ± 1.96 nmol/ μg protein/min for the control group ($p < 0.025$); 2.79 ± 2.17 nmol/ μg protein/min and 8.40 ± 2.36 nmol/ μg protein/min for the potentially allergic group ($p < 0.0025$); 0.92 ± 1.61 nmol/ μg protein/min and 5.72 ± 1.03 nmol/ μg protein/min for the allergic group ($p < 0.001$); and 1.08 ± 1.03 nmol/ μg protein/min and 5.88 ± 1.70 nmol/ μg protein/min for the asthmatic group ($p < 0.001$). There was a significant difference in prefreezing Na^+, K^+ ATPase activity between the allergic and asthmatic groups as compared with the control group ($p < 0.005$ for both). There was also a significant difference in postfreezing Na^+, K^+ ATPase activity between the potentially allergic, allergic, and asthmatic groups as compared with the control group ($p < 0.005$ for all).

In this study the potentially allergic subjects and the patients with asthma had significantly decreased methacholine AUCs as compared with the control subjects. The mean ($\pm$ SD) methacholine AUCs (percent baseline $\text{FEV}_1 \times \log [\text{mg/ml}]$) were 1168 ± 26 for the control group, 1066 ± 174 for the potentially allergic group ($p < 0.05$ as compared with the control group), 1122 ± 126 for the allergic group, and 966 ± 166 for the asthmatic group ($p < 0.001$) as compared with the control group). Indeed, a significant negative correlation ($r = -0.44$, $p < 0.005$) was detected between methacholine AUC (percent baseline $\text{FEV}_1 \times \log [\text{mg/ml}]$) and platelet mem-

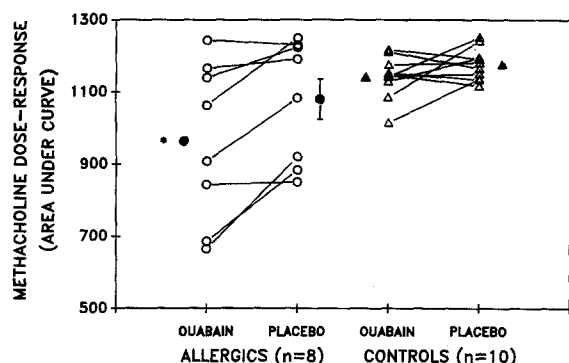


FIG. 2. Effect of ouabain versus placebo prechallenge on human bronchial methacholine responsiveness (AUC expressed as percent baseline $FEV_1 \times \log [mg/ml]$) in allergic ($n = 8$) and control ($n = 10$) subjects. * $p < 0.05$ compared with ouabain prechallenge in control subjects.

brane Na^+, K^+ ATPase inhibition (postfreezing – prefreezing Na^+, K^+ ATPase expressed as nmoles per microgram of protein per minute), such that high levels of AHR (low AUC) were associated with high levels of Na^+, K^+ ATPase inhibitory activity (Fig. 1).

The patients with allergy were subdivided into two groups on the basis of whether they were medicated (as described above) and the distribution of prefreezing and postfreezing platelet membrane Na^+, K^+ ATPase values, and methacholine AUCs were plotted (data not shown). There was no difference in the distribution of either parameter between the medicated and nonmedicated patients with allergy.

To test for a causal relationship between Na^+, K^+ ATPase inhibition and AHR, a second experiment was conducted to determine whether ouabain, a potent Na^+, K^+ ATPase inhibitor, directly influenced the level of methacholine AHR. Table I shows the methacholine PC_{20} for each subject after ouabain and placebo prechallenges. One allergic/asthmatic subject (subject 9) manifested 35% and 41% decreases in FEV_1 after prechallenge with placebo and ouabain, respectively; consequently, this subject was not tested for methacholine AHR after either of the prechallenges. Another allergic/asthmatic subject (subject 10) manifested a 37% reduction in FEV_1 after ouabain prechallenge only; again, this subject was not tested for methacholine AHR after ouabain prechallenge but was tested after placebo prechallenge. In addition to decreasing FEV_1 in one allergic/asthmatic subject, ouabain increased methacholine AHR in four other patients with allergy (subjects 4 to 7). Three of these four subjects manifested methacholine AHR after placebo prechallenge ($PC_{20} < 25$ mg/ml). Comparatively, all four subjects responded at one dose earlier in the methacholine challenge sequence after ouabain prechallenge. Ouabain had no effect in the other four patients with allergy (subjects 1 to 3 and 8). In contrast to the patients with allergy, none of the control subjects manifested decreases in FEV_1 after ouabain

TABLE I. Methacholine PC_{20} after ouabain and placebo prechallenges in patients with allergy

Subject No.	PC_{20} (mg/ml)	
	Ouabain	Placebo
1	>25	>25
2	>25	>25
3	>25	>25
4	11.7	>25
5	7.2	17.5
6*	0.88	5.5
7*	0.62	5.5
8	4.5	4.5
9*	ND	ND
10*	ND	ND

ND, No methacholine challenge.

*Patient with asthma.

inhalation, and none had AHR to methacholine after either placebo or ouabain prechallenges.

For each subject, an AUC for methacholine responsiveness was calculated and plotted for both ouabain and placebo prechallenges. Fig. 2 shows the effect of ouabain versus placebo prechallenge on bronchial methacholine responsiveness (AUC expressed as percent baseline $FEV_1 \times \log [mg/ml]$) in eight patients with allergy and 10 control subjects. Data from the two patients with allergy who experienced a reduction in FEV_1 of greater than 20% after placebo and/or ouabain inhalation were excluded from this analysis. Ouabain prechallenge increased methacholine AHR in the allergic group. Indeed, methacholine AHR was increased (lower AUC) in seven of the eight patients with allergy after ouabain versus placebo prechallenge. Mean AUCs ($\pm$ SEM) for the allergic group were 964 ± 91.0 and 1080 ± 56.0 after prechallenge with ouabain and placebo, respectively. The mean AUC for ouabain versus placebo prechallenge in the control group was 1142.2 ± 17.0 and 1176.5 ± 14.2 , respectively. The mean AUC after ouabain prechallenge was significantly different between the control subjects and patients with allergy ($p < 0.05$).

The patients with allergy were subdivided into two groups on the basis of whether they were medicated, and the distribution of AUCs for the ouabain and placebo prechallenges was plotted (data not shown). There was no difference in the distribution of AUCs between the medicated and nonmedicated patients with allergy. Next, to determine whether there was a correlation between Na^+, K^+ ATPase inhibition and bronchial responsiveness to ouabain, the difference between methacholine AHR (AUC expressed as percent baseline $FEV_1 \times \log [mg/ml]$) after the placebo and ouabain prechallenges was plotted as a function of the corresponding difference between postfreezing and prefreezing platelet membrane Na^+, K^+ ATPase activities (nmol/ μ g protein/min; Fig. 3). Data from the two patients with allergy who experienced a reduction of greater than 20% in

FEV₁ after placebo and/or ouabain inhalation were excluded from this analysis. As observed in the first experiment, the patients with allergy displayed large postfreezing increases in platelet membrane Na⁺,K⁺ ATPase activity in comparison with the control subjects. The respective mean ($\pm$ SD) prefreezing and postfreezing Na⁺,K⁺ ATPase activities were 5.17 ± 2.15 nmol/ μ g protein/min and 2.05 ± 1.26 nmol/ μ g protein/min for the control group ($p < 0.0025$) and 3.43 ± 3.14 nmol/ μ g protein/min and 4.82 ± 2.75 nmol/ μ g protein/min for the allergic group. Moreover, a significant positive correlation ($r = 0.49$, $p < 0.05$) was observed between the level of platelet membrane Na⁺,K⁺ ATPase inhibition and the level of bronchial responsiveness to ouabain.

DISCUSSION

The results of these studies document the existence of a correlative relationship between the level of platelet membrane Na⁺,K⁺ ATPase inhibition and the levels of airway responsiveness to methacholine and ouabain. The results also document that inhalation of ouabain, an Na⁺,K⁺ ATPase inhibitor, had significant effects on both airway caliber (FEV₁) and reactivity (methacholine AHR) in patients with allergy only. Collectively, these results provide evidence for a possible causal relationship between Na⁺,K⁺ ATPase inhibition and AHR.

Other investigators, using either in vitro or animal models of AHR, have demonstrated a role for Na⁺,K⁺ ATPase activity in determining smooth muscle contractility. Souhrada et al.¹ reported the presence of an electrogenic Na⁺ pump in airway smooth muscle isolated from bovine and guinea pig trachea and further demonstrated that in vitro ouabain administration induced a significant contraction in airway smooth muscle from both sources. Another study by Agarwal and Hyatt¹⁰ demonstrated that ouabain caused bronchoconstriction and increased histamine responsiveness in guinea pigs in vivo. More recently, Knox et al.⁶ showed that ouabain increased bovine and human airway smooth muscle contractility in vitro.

However, in follow-up studies, Knox et al.²⁵ failed to demonstrate that in vivo treatment with either inhaled ouabain or oral digoxin, another cardiac glycoside, had any significant effect on human AHR. These findings are in agreement with the results of other investigators²⁶ but are in conflict with those of this study, in which in vivo ouabain inhalation increased methacholine AHR in patients with allergy. Possible explanations for this discrepancy include differences in: (1) patient characteristics, (2) the dosage and administration of ouabain, (3) the length of time between ouabain prechallenge and AHR provocation, and (4) the chemical used for provocative testing after ouabain inhalation (histamine was used in the study by Knox et al.²⁵).

Although ouabain inhalation increased methacholine AHR in patients with allergy in this study, there is no evidence demonstrating the mechanism by which this occurred. It is possible that ouabain increased AHR through direct inhibition of airway smooth muscle

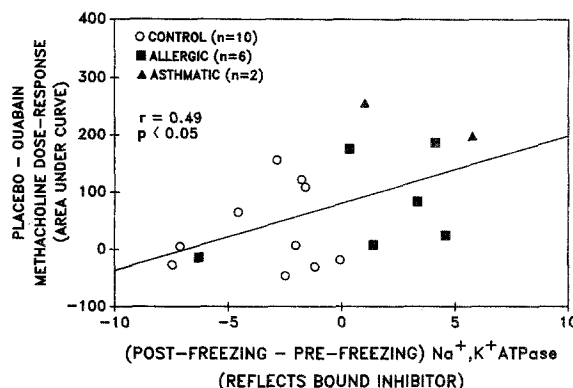


FIG. 3. Correlation between platelet Na⁺,K⁺ ATPase inhibition (postfreezing - prefreezing Na⁺,K⁺ ATPase expressed as nanomoles per microgram of protein per minute) and bronchial responsiveness to ouabain (AUC expressed as percent baseline FEV₁ $\times$ log [mg/ml]) in 18 subjects.

Na⁺,K⁺ ATPase; however, an effect on another cell type cannot be ruled out. For example, under certain conditions, inhibition of Na⁺,K⁺ ATPase activity can enhance histamine release from mast cells.^{27,28} An increase in the local histamine concentration could conceivably enhance airway smooth muscle contractility. Likewise, ouabain could have exerted its effect by modulating the responsiveness of other inflammatory cells or even nerve cells, which are present in the airways.

To obtain the correlative results presented here, a proinflammatory cell, the blood platelet, was used to examine the extent of cellular Na⁺,K⁺ ATPase inhibition. A correlation with airway responsiveness to both methacholine and ouabain was noted. The blood platelet was used because it is readily accessible, and its Na⁺,K⁺ ATPase activity has been demonstrated to be similar to that of other inflammatory (leukocytes) and noninflammatory (smooth muscle cells) cell types, which may be involved in the pathogenesis of AHR.²⁹ Additionally, the blood platelet is being increasingly recognized as an inflammatory cell that may contribute to AHR pathogenesis. Indeed, platelets release numerous autocooids and mediators that initiate various aspects of the inflammatory response or act directly on airway or vascular smooth muscle; and platelets interact with other inflammatory and noninflammatory cell types through their release of mediators, chemoattractants, and growth factors.³⁰⁻⁴³ Finally, because they share many characteristics with smooth muscle cells, including an α_2 -adrenoreceptor-cyclase system and a calcium-dependent contraction-coupling mechanism, human platelets have been extensively used as a model in the study of hypertension and, more recently, AHR.⁴⁴⁻⁵²

The biochemical defect in AHR has not been defined; moreover, the role of a potential endogenous Na⁺,K⁺ ATPase inhibitor in AHR is unknown. However, several indirect lines of evidence suggest that an Na⁺,K⁺ ATPase inhibitor could represent a biochemical defect, which is involved in the pathogenesis of AHR. The

potential consequences of a reduction in Na^+, K^+ ATPase activity include an alteration of electrical membrane potential¹⁴ and increases in intracellular Na^+ concentration, which can increase intracellular Ca^{++} concentrations.¹⁵ In addition, studies, in which the transmission of β -adrenergic receptor signals was examined, have implicated a role for activation of the Na^+, K^+ ATPase pump in the relaxation of airway smooth muscle.¹⁶ Inhibition of Na^+, K^+ ATPase activity could conceivably downregulate any pre-relaxation stimuli transmitted through the β -adrenergic receptor and, consequently, create a procontractile state. The level of AHR could potentially be increased through any or all of these mechanisms.^{16, 17, 18} Moreover, recent epidemiologic studies have suggested a possible role for an endogenous Na^+, K^+ ATPase inhibitor in human AHR.²⁻⁵ These studies suggest that a high Na^+ diet and subsequent extracellular volume expansion lead to increased production of a circulating Na^+, K^+ ATPase inhibitor, which could increase airway smooth muscle contractility by mechanisms described above. Another possibility is that an Na^+, K^+ ATPase inhibitor could influence the activity of local inflammatory cells, including mast cells,^{27, 28} and contribute to AHR through an increase in airway inflammation.

Although this study examined the effect of inhaled ouabain on AHR, it is conceivable that an endogenous Na^+, K^+ ATPase inhibitor could similarly affect AHR. This is supported by the correlative relationship observed between the levels of platelet Na^+, K^+ ATPase inhibition (caused by endogenous inhibitor) and AHR. Because of the wide distribution of Na^+, K^+ ATPase, an endogenous inhibitor might be expected to yield extensive biologic effects. However, an endogenous inhibitor in AHR could have its origin and primary pharmacologic action locally in the same tissue, where high levels may be expected. In AHR, the site would likely be the airways or pulmonary vasculature. Consequently, any inhibitor that spills over into plasma may not be in sufficient concentration to affect the functioning of other important tissues, such as the heart, which is known to be affected by cardiac glycosides. Alternatively, isoenzymes and receptor affinities for the endogenous inhibitor may vary from tissue to tissue, thus potentially explaining the effect of Na^+, K^+ ATPase inhibitor on one tissue and not another.⁵³

Conversely, oral digoxin therapy used in the treatment of cardiac disease could potentially have the same effect on AHR as inhaled ouabain. However, oral cardiac glycosides have not been reported to increase AHR. It is possible that orally administered digoxin does not achieve high enough local concentrations to alter AHR. Alternatively, receptors for the endogenous inhibitor may have a decreased affinity for digoxin.

In this study the bronchial reactivity of patients with allergy was more sensitive to ouabain inhalation than that of control subjects. One possible explanation is that patients with allergy, as compared with control subjects, may possess a unique Na^+, K^+ ATPase isoenzyme with a

higher binding affinity for ouabain and even an endogenous inhibitor.⁵³ Alternatively, in patients with allergy, a fraction of the ouabain receptors may be preoccupied by an endogenous Na^+, K^+ ATPase inhibitor. In subjects possessing a prebound endogenous inhibitor, it is conceivable that lower concentrations of exogenous inhibitor would be required to alter AHR. Finally, it is conceivable that ouabain inhalation increased AHR in control subjects but that the parameter used to assess AHR (PC_{20}) was insensitive to these changes.

In these studies the subset of patients with allergy using theophylline and/or a β -adrenoceptor agonist MDI as needed did not differ from the nonmedicated patients with allergy with respect to prefreezing and postfreezing platelet membrane Na^+, K^+ ATPase levels. These results are consistent with those from previous studies conducted in our laboratory with the platelet model.^{11, 12}

Finally, although the freezing process was associated with an increase in the Na^+, K^+ ATPase activity levels of patients with allergic diseases, it was associated with a decrease in the Na^+, K^+ ATPase activity of the control subjects. This observation has been previously reported and is not unexpected because freezing may lead to enzyme degradation.^{11, 12}

In summary, these data provide two separate lines of evidence that inhibition of Na^+, K^+ ATPase activity increases AHR and support the possibility that an endogenous inhibitor of Na^+, K^+ ATPase could play a role in the pathogenesis of AHR in human beings.

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