

Evaluation of the gut mucosal barrier: Evidence for increased antigen transfer in children with atopic eczema

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Background: Intestinal antigen handling determines subsequent immune response to the antigen. Antigens are absorbed across epithelium along two functional pathways. The main pathway is degradative, which reduces the immunogenicity of the antigen. A minor pathway allows the transport of intact proteins, which is crucial for antigen-specific immune responses. The Ussing chamber method allows the quantitative measurement of protein transfer across the intestinal mucosa.

Objective: This study was designed to explore the theory that altered antigen transfer across the intestinal mucosa is a factor in the pathogenesis of atopic eczema, characterized by hyperreactivity to environmental antigens.

Methods: The absorption and degradation of horseradish peroxidase (molecular weight, 40,000 d) were studied in vitro in Ussing chambers. Eighteen biopsy specimens of upper small intestinal mucosa from 14 patients (aged 0.5 to 8 years) with atopic eczema and 18 specimens from 15 age-matched control subjects were examined.

Results: The mean (95% confidence interval) absorption of intact horseradish peroxidase was significantly higher in children with atopic eczema than in control subjects: 242 (81-404) $\text{pmol} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$ versus 23 (12-33) $\text{pmol} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$; $t = 2.86$, $p = 0.007$. The absorption of degraded horseradish peroxidase was 972 (732-1213) $\text{pmol} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$ in patients with atopic eczema and 672 (532-811) $\text{pmol} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$ in control subjects; $t = 2.29$, $p = 0.03$.

Conclusions: Our results may reflect a primarily altered antigen transfer in patients who have atopic eczema, which may initiate and perpetuate prompt immune responses to common environmental antigens, including foods. (J ALLERGY CLIN IMMUNOL 1996;97:985-90.)

Key words: Intestinal absorption, atopic eczema

The cause of atopic eczema is unknown, but it is clear that hereditary predisposition is one of the most important factors in this chronically recurring skin disease.¹ Environmental antigens may initiate the disorder and contribute to its persistence. The route of sensitization varies with age: early in life dietary antigens predominate.² Evidence that food allergy is implicated in atopic eczema is afforded by the clinical benefit of following exclusion diets for infants with eczema.³ High-level allergen exposure

Abbreviations used

CI:	Confidence interval
HRP:	Horseradish peroxidase
I_{sc} :	Short-circuit current

during the first few months of life may predispose children to allergic sensitization.⁴

The intestinal mucosa is an important organ of defense, providing a barrier against the antigens encountered by the enteric route.⁵ The barrier functions may be incompletely developed in early infancy, which may explain the peak prevalence of food allergies in this age group. In attempts to correlate atopic eczema with impaired gut mucosal barrier functions, both increased intestinal permeability⁶⁻¹⁰ to low-molecular-weight probes (e.g., lactulose, mannitol, rhamnose) and normal intestinal permeability¹¹⁻¹³ have been reported. Al-

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though intestinal permeability to low-molecular-weight probes and antigen absorption represent different phenomena, they have been shown to alter in the same direction.^{14, 15} The mode of antigen absorption across the intestinal mucosa has been shown to exert a significant influence on the immune response to the antigen in question.¹⁶ The analysis of antigen processing and uptake by the gut remains a challenge because of the potential technical difficulties, antigen-antibody interactions, and rapid clearance at the mucosal and systemic levels.¹⁷ For the first time we have assessed antigen transfer across intestinal mucosa in children with atopic eczema. For this purpose, horseradish peroxidase (HRP) (molecular weight, 40,000 d) was selected as the tracer protein. The structure of HRP corresponds to that of dietary antigens,¹⁸ and its absorption pattern is well characterized¹⁹ and consistent throughout the small intestine.²⁰ The absorption of HRP *in vitro* in the healthy small intestine and in various diseased states correlates with that of different antigenic macromolecules²⁰⁻²² and with its absorption *in vivo*.^{23, 24}

METHODS

Patients and study design

The study was carried out from 1989 to 1994. A total of 29 children were examined. Eighteen biopsy specimens of upper small intestinal mucosa from 14 patients (11 male and 3 female, aged 0.5 to 8 years) with atopic eczema were investigated.

The patients with atopic eczema fulfilled Hanifin's criteria²⁵ for atopic eczema in children. A positive family history of atopic diseases (asthma, atopic eczema, and allergic rhinitis) or food allergy in first-degree relatives was noted in 12 of 14 (86%) patients. The eczematous lesions were treated with emollients and topical corticosteroids. No patient was receiving systemic corticosteroid therapy. In addition to atopic eczema, gastrointestinal disturbances, such as loose stools, vomiting, or diarrhea, were seen in two patients; and respiratory symptoms, such as wheezing or asthma, were seen in three. All patients underwent biopsy to confirm that mucosal histologic findings were normal before the diet was broadened.

Prick tests for dietary antigens were done with commercially available allergens (Allergologisk Laboratorium A/S, Horsholm, Denmark). Reactions were read at 15 minutes, and half of the histamine reaction size or more was recorded as positive on condition that the mean diameter of the wheal was at least 3 mm, and the negative control at the same time, 0 mm. Serum total IgE, antigen-specific IgE (RAST; Pharmacia, Uppsala, Sweden) for cow's milk, egg, or cereals was determined in the study patients.

Double-blind, placebo-controlled cow's milk challenge protocol was performed as described previously.²⁶ The placebo formula was the synthetic amino acid-derived Neocate (SHS International Ltd., Liverpool, U.K.), and the test formula consisted of Neocate and 10 gm of cow's milk powder/100 ml. The challenge period for each formula was 7 days. On the first day of the oral challenge with placebo or test formula, increasing doses (1, 5, 10, 20, 50, and 100 ml) were given at 30-minute intervals until milk intake appropriate for age was reached. If no reaction occurred within 8 hours in the hospital, the challenge was continued at the patient's home. When a clinical reaction appeared, the patient was examined in the hospital, and the challenge was discontinued. All patients were examined on days 8 and 15 of the challenge, corresponding to the placebo and cow's milk challenge period, and thereafter the code was opened. Cow's milk allergy was defined as an unequivocally adverse reaction to cow's milk challenge.

Eighteen biopsy specimens from 15 children (aged 0.5 to 8 years), who served as a control group, were studied. These patients had been referred for small intestinal biopsy because of symptoms or serologic test results suggestive of intestinal disease. All patients proved to be healthy, and no disease was found in these examinations.

The study protocol was approved by the Ethical Review Committee of Tampere University Hospital. Informed consent was obtained from the parents of patients and control subjects.

HRP absorption studies

Biopsy specimens, taken by endoscopic technique, from mucosa of the distal duodenum/upper jejunum were studied in an Ussing chamber (exposed area, 0.05 cm²). An adjacent sample was taken for histologic examination. The Ussing chamber method allows the quantitative measurement of protein transfer with determination of protein transfer in its intact and degraded forms.¹⁹ The integrity of the tissue during the experiment can be monitored by constant recording of the electrical parameters: transepithelial potential difference, total ionic conductance, and short-circuit current (I_{SC}). Potential difference across an intestinal epithelium is normally generated by the Na^+ , K^+ -adenosine triphosphatase in the basolateral membranes of the mature villi; whereas total ionic conductance reflects the integrity of the tight junctions, and I_{SC} reflects active electrolyte transport capacities.

The tissues were washed on both sides with Ringer's solution (pH 7.4) containing: 140 mmol/L Na^+ , 5.2 mmol/L K^+ , 1.2 mmol/L Ca^{2+} , 1.2 mmol/L Mg^{2+} , 120 mmol/L Cl^- , 25 mmol/L HCO_3^- , 2.4 mmol/L HPO_4^{2-} , and 0.4 mmol/L $H_2PO_4^-$. The solution was oxygenated (95% O_2 ; 5% CO_2), circulated, and maintained at 37° C. The solutions in which the mucosal and serosal sides were washed were connected by 3 mol/L KCl bridges to calomel electrodes for measurement of the potential difference. Fluxes were measured under open-circuit

conditions. Total ionic conductance was assessed by a 10 μ A current passing regularly through the system via Ag-AgCl electrodes. The I_{SC} was then calculated, taking into account fluid and filter resistance.

HRP (Sigma type VI; Sigma Chemical Co., St. Louis, Mo.) was added to the mucosal compartment at a final concentration of 0.4 mg/ml to quantitate the absorption of the intact protein from mucosal to serosal side. Serial 800 μ l samples were taken from serosal side, the first at 10 minutes after commencement of the experiment and the next at 20-minute intervals for up to 90 minutes. This fluid volume was replaced by fresh Ringer's solution. The amount of intact protein appearing in the serosal compartment was assessed by measuring the enzymatic activity of HRP in 200 μ l aliquots of serosal samples. H_2O_2 was used as the substrate and o-dianisidine as the dye. The enzymatic activity of intact HRP in samples from the serosal side was determined with a spectrophotometer and compared with the enzymatic activity of initial known HRP concentration to obtain the absorption of HRP in picomoles. The specific activity of HRP, which was measured before and after the experiment, remained constant. Previous studies have shown that HRP, measured enzymatically after absorption, shows a gel elution pattern of native HRP,²⁷ and only intact HRP has enzymatic activity.¹⁹

Iodinated HRP (¹²⁵I-HRP) was used as a tracer for quantitation of the total protein absorption. HRP was iodinated (500 μ Ci/1.0 mg HRP) according to the method of Markwell.²⁸ Labeled HRP was separated from free iodide by gel filtration. Iodinated HRP was added to the mucosal side, and total HRP absorption was assessed by counting the radioactivity of the 500 μ l aliquots from the samples taken from serosal side of the Ussing chamber. By using high-performance liquid chromatographic SuperDex gel filtration (Pharmacia Diagnostics, Uppsala, Sweden), it was confirmed that there was no free labeling and that the radioactivity was fixed either to the intact or degraded fragments after crossing the intestinal mucosa. Degraded HRP absorption was then calculated by subtracting intact HRP absorption from total protein absorption. Peroxidase activity appeared in the serosal side 30 minutes after the addition of HRP to the mucosal side.

The rates of HRP absorption remained constant between 50 and 90 minutes after HRP addition. The absorption is therefore reported as a mean of the measurements made during this steady-state period.

Statistics

Means are presented with 95% confidence intervals (CIs). Student's two-tailed *t* test was used in statistical comparisons.

RESULTS

Clinical data

The mean (95% CI) serum total IgE was 514 (175-1509) kU/L. RAST for cow's milk was posi-

tive (>0.4 kU/L) in 12 of 14 (86%) patients with atopic eczema. Results of skin prick tests with cow's milk were positive in nine of 11 (82%) patients. On the basis of results of skin prick tests, RAST, and double-blind placebo-controlled cow's milk challenges, 10 patients had dietary restrictions for cow's milk, 10 for egg, and nine for cereals (wheat, barley, rye, and oats). All patients had dietary restrictions for various fruits and vegetables. Two patients had been on a cow's milk elimination diet but had become tolerant to cow's milk. At the time of biopsy, all patients were on a tolerated diet. Histologic findings in the biopsy specimens were normal.

HRP transfer

The mean (95% CI) absorption of intact HRP was significantly higher in children with atopic eczema ($n = 14$), 242 (81-404) $\text{pmol} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$, than in control subjects ($n = 15$), 23 (12-33) $\text{pmol} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$; $t = 2.86$, $p = 0.007$ (Fig. 1). The mean absorption of degraded HRP across the tissue samples was 972 (732-1213) $\text{pmol} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$ in atopic patients and 672 (532-811) $\text{pmol} \cdot \text{hr}^{-1} \cdot \text{cm}^{-2}$ in control subjects; $t = 2.29$, $p = 0.03$ (Fig. 1). The mean fraction absorbed intact was 20% in the patients with atopic eczema and 3% in the control subjects.

Electrical parameters

The electrical parameters were comparable and within physiologic limits in both groups. The mean (95% CI) total ionic conductance, which reflects the integrity of the tight junctions, was 68 (40-98) mS/cm^2 in patients with atopic eczema and 53 (33-73) mS/cm^2 in control subjects; $t = 0.95$, $p = 0.35$. The I_{SC} , which reflects active electrolyte transport capacity, was 42 (22-59) $\mu\text{A/cm}^2$ in patients with atopic eczema and 29 (10-50) $\mu\text{A/cm}^2$ in control subjects; $t = 1.0$, $p = 0.30$.

DISCUSSION

The intestinal mucosa has two opposing functions: to provide a barrier that excludes numerous harmful antigens derived from microorganisms and food and to transfer antigens essential for evoking specific immune responses. Most macromolecules, such as dietary antigens, are excluded by the intestine's mucosal barrier.⁵ The integrity of the defense barrier is necessary to prevent inappropriate and uncontrolled antigen transport. However, even in physiologic conditions, a quantitatively unimportant but immunologically important fraction of antigens bypasses this barrier and is

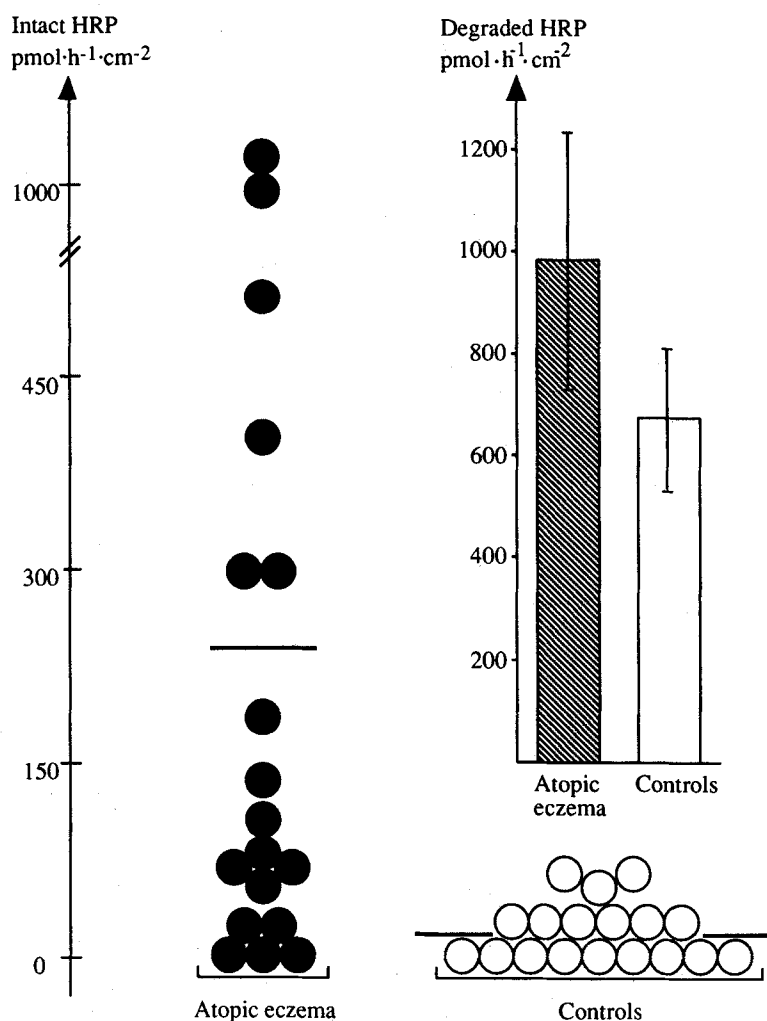


FIG. 1. Absorption of intact and degraded HRP across upper small intestinal specimens from patients with atopic eczema and control subjects.

thus available for intestinal absorption, which is crucial for antigen-specific immune responses.

Dietary antigens are macromolecules with a molecular weight in the range of 10,000 to 70,000 d.¹⁸ They are absorbed across the epithelial layer by transcytosis along two functional pathways.²⁹ The main degradative pathway entails lysosomal processing of the protein to smaller peptide fragments, which reduces immunogenicity of the protein and is important in host defense to diminish the antigen load. More than 90% of the protein internalized passes in this way. A minor pathway allows the transport of intact proteins, which results in antigen-specific immune responses.¹⁶ In health, paracellular leakage of macromolecules is not allowed because intact intercellular tight junctions maintain the macromolecular barrier. Consequently, in health, antigen transfer is well con-

trolled, and aberrant antigen absorption does not occur.

Determination of both intact and degraded antigen absorption is important because they can be affected separately,²² and their clinical and immunologic consequences may be different. Antigen handling in the gut is associated with the generation of oral tolerance.³⁰ There is evidence that during the absorption process antigens are subtly altered into tolerogenic form.³¹ In the immature gut, because of immature absorptive functions, antigen exposure may result in priming for immune responses instead of oral tolerance.³² Increased uptake of intact food antigens in the immature gut has been explained by increased binding of antigens to the microvillus membrane.³³ Aberrant and excessive antigen absorption increases the antigen load, which may be harmful to

the host. In a like manner, incomplete degradation may result in the generation of new antigenic epitopes.⁵ In this study we found increased protein absorption, in intact and degraded form, in children with atopic eczema. Because the degraded protein transport must be transcellular, our finding may reflect an intrinsic defect in epithelial cells and their degradative functions.

It is not known whether altered antigen transfer is a primary or secondary phenomenon in atopic eczema. In food allergy, disturbances in intestinal permeability³⁴ and antigen transfer³⁵ occur when an allergen comes into contact with the intestinal mucosa. It has previously been shown that in active cow's milk allergy with predominantly gastrointestinal symptoms the absorption of both intact and degraded HRP is increased in untreated cases, but after complete avoidance of cow's milk, HRP transport returns to normal.³⁵ The results are in accordance with those of our group when a lactulose-mannitol test was used to measure gut permeability in children with cow's milk allergy; a significant increase in lactulose-mannitol excretion ratios followed a positive cow's milk challenge response in patients manifesting cutaneous and gastrointestinal reactions alike.³⁴ Intestinal hypersensitivity reactions in sensitized experimental animals have been associated with an increased uptake of antigens and an increased lactulose-rhamnose excretion ratio.¹⁴ These observations would suggest that in cow's milk allergy, enhanced antigen transfer is secondary to an abnormal intestinal immune response to cow's milk antigens. By contrast, in our patients on elimination diet, the alterations in antigen transfer were similar to those of patients with cow's milk allergy before elimination diet was started.

In our patients, the offending foods were identified and eliminated; that is, the patients were not challenged in vivo. Mucosal histologic findings were normal in all specimens. We avoided challenging our patients with atopic eczema in vitro and therefore used HRP as a tracer of antigen absorption, instead of cow's milk proteins or ovalbumin. We found no modifications in I_{SC} , which classically correlates with immediate mucosal hypersensitivity reactions to food proteins,^{36,37} and we found no changes in the total ionic conductance, which indicates integrity of the tight junctions.³⁸ On this basis, our results may reflect a primarily altered antigen transfer in patients who have atopic eczema. Aberrant antigen absorption could partly explain why patients who have atopic eczema frequently show prompt immune re-

sponses to common environmental antigens, including dietary antigens.

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