

Enhanced Transepithelial Antigen Transport in Intestine of Allergic Mice Is Mediated by IgE/CD23 and Regulated by Interleukin-4

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Background & Aims: We previously described a system for enhanced transepithelial transport of antigen in which both the amount of specific antigen and its rate of transport were dramatically increased in intestine of sensitized rats compared with controls. This study investigated the essential components mediating antigen uptake in mice genetically deficient for interleukin (IL)-4 or CD23. **Methods:** Mice were actively or passively sensitized to horseradish peroxidase (HRP). Jejunal segments from control or sensitized mice were mounted in Ussing chambers and challenged with HRP from the luminal side. Tissues were processed for electron microscopy, and photomicrographs were analyzed for antigen uptake (location and area of HRP-containing endosomes). Immunohistochemistry and reverse-transcription polymerase chain reaction were used to detect epithelial CD23 expression. **Results:** Actively sensitized IL-4^{+/+}, but not IL-4^{-/-} mice, displayed increased transepithelial antigen transport and CD23 expression on enterocytes. Passively sensitized IL-4^{+/+} and IL-4^{-/-} mice displayed elevated antigen transport after transfer of immune serum but not if the serum was depleted of immunoglobulin (Ig) E or IL-4. IL-4 added to cultured IEC-4 cells up-regulated expression of CD23 messenger RNA. The augmented antigen uptake was inhibited by anti-CD23 and was absent in sensitized CD23^{-/-} mice. **Conclusions:** Our studies indicate that IL-4 regulates IgE/CD23-mediated enhanced transepithelial antigen transport in sensitized mouse intestine.

Allergic diseases affect 20%–30% of the population in the United States, and their prevalence is increasing in developed countries.¹ Food allergy is one type of allergic disorder that involves 2%–5% of adults and an even greater proportion of children.² Sensitized individuals develop gastrointestinal symptoms, such as nausea, vomiting, and diarrhea, and in some cases respiratory and cutaneous symptoms, after ingesting specific foods. In severe cases, such as peanut allergy, systemic anaphylaxis

can occur with fatal consequences. The onset of hypersensitivity reactions in humans and animal models of food allergy is extremely rapid (within minutes) after the exposure to antigens.^{3–5} The rapid nature of the reactions has resulted in the use of the term “immediate” hypersensitivity. However, the mechanism accounting for the rapid response has never been completely explained.

It is generally accepted that intestinal anaphylactic reactions are caused by biological mediators released from mucosal mast cells after antigen cross-linking of immunoglobulin (Ig) E bound to the cell surface.⁵ However, macromolecular food antigens must first cross the intestinal epithelial barrier before gaining access to mast cells in the subepithelial compartment. This barrier is composed of a single layer of cells, mainly enterocytes, linked by tight junctions that restrict molecules larger than 500 daltons from passing through the paracellular pathways.⁶ Enterocytes themselves have the capacity to transport a small proportion of antigenic material from the intestinal lumen to the underlying tissues via transcytosis, a process that takes ~20–30 minutes.⁷ However, most protein antigens are digested either in the lumen or by lysosomal enzymes inside the cell. Therefore, most food proteins lose their antigenic properties before or during transepithelial transport. In contrast, we recently reported our findings of greatly enhanced transepithelial transport of intact antigen in less than 2 minutes in an experimental rat model of food allergy.^{8–11}

We previously examined the route and timing of intestinal transepithelial antigen transport in sensitized

Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IEC, intestinal epithelial cell; IL, interleukin; OVA, ovalbumin; PCA, passive cutaneous anaphylaxis; PCR, polymerase chain reaction; RT, reverse-transcription.

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0016-5085/01/\$35.00

doi:10.1053/gast.2001.26470

rats using horseradish peroxidase (HRP) as a model antigen.⁸ HRP was used because the intact molecule can be accurately measured and its reaction product can be visualized in tissues. We showed that both the quantity of antigen taken up by epithelial cells and its rate of transport into the lamina propria were increased several-fold in sensitized rats compared with naive control rats. Electron microscopy indicated that the route of transepithelial antigen transport during the initial phase (phase I) was intracellular via endosomes. Once the antigen was released into the lamina propria and mast cells were activated, a second phase (phase II) of antigen transport occurred involving recruitment of the paracellular pathway. Phase II augmented permeability, allowing for even greater antigen uptake. Phase I was shown to be mast cell-independent, occurring before the antigen-induced ion secretion caused by release of mast cell mediators, but specific for the antigen to which the rats were sensitized.^{8,9} Phase II required mast cells because it did not occur in mast cell-deficient rats.^{9,10}

Our finding that enhanced antigen transport across enterocytes in sensitized rats was antigen specific suggested an Ig recognition mechanism at the level of the epithelium. We identified that sensitization induced the expression of the low-affinity IgE receptor, CD23/FcεRII, on epithelial cells in the rat jejunum.¹¹ In addition, after challenge with HRP antigen to the luminal surface of intestinal tissues, the receptor was internalized and was localized on the membrane of enterocyte endosomes containing HRP. Importantly, anti-CD23 antibody inhibited phase I antigen uptake leading to the reduction of the hypersensitivity reaction and phase II antigen transport in rat intestine.¹¹

The aim of the present study was to identify whether the phase I-enhanced transepithelial antigen transport is present in another species and to investigate the essential components involved in the mechanism of this augmented antigen uptake system using genetic-deficient mice. Interleukin (IL)-4 is a major isotype switching factor for IgE synthesis in B lymphocytes,¹² and increased IL-4 production in allergic patients has been reported.^{13,14} IL-4 also plays a role in up-regulating the expression of IgE receptors, including CD23, on B cells.¹⁵⁻¹⁸ Therefore, we designed experiments to examine the role of IL-4 in epithelial CD23 expression and the consequence of genetic deletions for IL-4 and CD23 on phase I transepithelial antigen transport. Our studies indicate the following: (1) the phenomenon of enhanced intestinal transepithelial antigen transport is reproducible in sensitized mice, (2) IgE/CD23 mediates the augmented antigen transport in enterocytes, and (3) IL-4

plays a major regulatory role in the mechanism through both stimulation of IgE synthesis and up-regulation of CD23 expression on intestinal epithelial cells.

Materials and Methods

Animals

Adult male IL-4^{+/+} and IL-4^{-/-} mice and CD23^{+/+} and CD23^{-/-} mice were used in this study. IL-4^{-/-} mice (BALB/c background) were from a colony maintained in the Central Animal Facility at McMaster University (initially obtained from H. Bluethmann, Pharmaceutical Research Gene Technology, F. Hoffmann-La Roche, Basel, Switzerland). IL-4^{+/+} (BALB/c) mice were purchased from Harlan Breeding Laboratories, Indianapolis, IN. IL-4^{+/+} and IL-4^{-/-} mice were actively sensitized by intraperitoneal injection with 100 μg HRP (type II; Sigma Chemical Company, St. Louis, MO) or ovalbumin (OVA) (Grade V; Sigma) in 0.2 mL of alum, and 0.1 mL of pertussis vaccine (Connaught Laboratories, Willowdale, Ontario, Canada) as adjuvants.⁴ Experiments were conducted on day 14.

CD23^{-/-} mice (C57BL/6 background) were initially obtained from M. Lamers, Max-Planck-Institut für Immunbiologie, Freiburg, Germany.¹⁹ CD23^{+/+} (C57BL/6) mice were purchased from Harlan Breeding Laboratories, Indianapolis, IN. C57BL/6 mice required boosting to induce active sensitization. CD23^{+/+} and CD23^{-/-} mice were injected with HRP as above except that 50 ng pertussis toxin²⁰ was substituted for vaccine and mice were boosted with HRP on days 7 and 14. Experiments were conducted on day 21.

Mice (IL-4^{+/+} and IL-4^{-/-}) were passively sensitized by intraperitoneal injection of 0.5 mL of high-IgE containing serum (titer 1:1024) obtained from actively sensitized Brown Norway rats, a high responder strain. The serum was either untreated or depleted of IgE by passing the serum through a column containing sepharose 4B beads (Pharmingen, Mississauga, ON, Canada) conjugated to anti-IgE (MARE-1, anti-rat IgE heavy chain; Serotec, Oxford, England). In some experiments, the serum was incubated with a neutralizing anti-IL-4 antibody (polyclonal rabbit anti-rat; Biosource, Camarillo, CA) for 1 hour at room temperature. Experiments were conducted 3 days after passive sensitization. In all studies, nonsensitized naive mice served as controls. The experiments described were conducted with approval from the McMaster University Animal Care Committee.

Ussing Chambers

Mice were killed by cervical dislocation and a laparotomy was performed. Four pieces of midjejunum were mounted in Ussing Chambers (WPI, Narco Scientific, Mississauga, ON, Canada). Care was taken to avoid Peyer's patches. An area of 0.6 cm² of intestine was exposed to 8 mL of circulating oxygenated Krebs buffer (pH 7.33–7.37) at 37°C. The serosal buffer contained 10 mmol/L glucose osmotically balanced by 10 mmol/L mannitol in the luminal buffer. The tissue was clamped at 0 V using a W-P Instruments automatic voltage

clamp (Narco Scientific). The short-circuit current (I_{sc} , $\mu A/cm^2$) was recorded on line. At 5-minute intervals, the tissue was pulsed with 1 mV for a duration of 1 second. The change of I_{sc} caused by the pulse was used to determine the tissue conductance (mS/cm^2) according to Ohm's law. Preliminary experiments indicated that baseline I_{sc} and conductance values of tissues from each group of mice were similar. Therefore, consistent baseline electrophysiological values and the I_{sc} responses to electrical transmural stimulation (10 Hz, 10 mA, 0.5 ms for a total time of 5 seconds) were used to confirm tissue viability. HRP antigen was added to the luminal buffer at 5×10^{-5} mol/L.

Epithelial Uptake and Transport of Antigen

Tissues were removed from the Ussing chamber 2 minutes after HRP antigen addition to the luminal buffer to examine HRP transport across the epithelium.⁸⁻¹¹ Jejunal segments were fixed in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4), washed, incubated in 3,3'-diaminobenzidine tetrahydrochlorine (Sigma) and H_2O_2 (pH 7.6), and subsequently processed for transmission electron microscopy. Ultrathin sections of midvillus epithelium (cut in the longitudinal plane) were stained, and photomicrographs were taken at a magnification of 3000. The total area of HRP-containing endosomes in enterocytes ($120 \mu m^2$ apical window) was quantified in photomicrographs using computerized image analysis.⁸⁻¹¹ In addition, the distribution of HRP-containing endosomes within enterocytes was determined and expressed as the percentage of cells containing endosomes in the apical, mid, or basal regions of enterocytes and in the lamina propria. This analysis was performed for 100 photomicrographs containing well-oriented enterocytes (tissues from 4 mice per group) by one investigator (P.C.Y.) who was unaware of the origin of the tissues. In some experiments, the effect of adding the well-characterized anti-CD23 antibody (B3B4, rat IgG2a anti-mouse CD23)²¹⁻²³ to the mucosal buffer 30 minutes before antigen challenge was examined. B3B4 was isolated from the supernatant of cultured hybridoma cells²¹ by ammonium sulfate precipitation followed by affinity purification in a protein G column (Mab Trap G II, Pharmingen). The control for the anti-CD23 was an isotype-matched irrelevant antibody purchased from Rockland Immunochemicals, Gilbertsville, PA.

Epithelial CD23 Protein Expression

A 1.5-cm segment of jejunum was snap frozen in optimal cutting temperature (OCT) solution (Sukura Co., Torrance, CA), cut into 5- μm sections, and processed for immunohistochemistry.¹¹ Endogenous peroxidase was quenched by treating sections with 0.01% H_2O_2 . CD23 expression was identified by the biotin-streptavidin method using B3B4 as the primary antibody at a dilution of 1:1000. The secondary antibody, biotinylated mouse anti-rat IgG (1:300) and streptavidin-HRP (1:600) were purchased from DAKO, Carpinteria, CA. Spleen tissues served as positive controls. Negative con-

trols included sections where the primary antibody was omitted or replaced with the isotype control antibody.

Epithelial CD23 Messenger RNA Expression

Mouse small intestinal epithelial cells of the IEC-4 cell line²⁴ were cultured in Dulbecco's modified Eagle medium (GIBCO BRL, Gaithersburg, MD) supplemented with 5% fetal calf serum, 0.01 mol/L HEPES, 20 mmol/L L-glutamine, 0.1 U/mL penicillin G, and 5 $\mu g/mL$ streptomycin. Confluent cells were treated with 10 ng/mL recombinant mouse IL-4 (R&D Systems, Minneapolis, MN) for various periods of time. The RNA was extracted from cells using an RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) and 2 μg RNA was reverse-transcribed by Oligo d(T)₁₆ using Perkin-Elmer RNA polymerase chain reaction (PCR) core kit (Perkin-Elmer, Mississauga, ON, Canada). The resulting complementary DNA (20 μL) was then subjected to PCR by addition of 80 μL of a master mix containing 1.5 mmol/L (for CD23 primers) or 2 mmol/L (for glyceraldehyde-3-phosphate dehydrogenase [G3PDH] primers) of $MgCl_2$ solution, $1 \times$ PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3), 2.5 U AmpliTaq DNA polymerase, 0.5 $\mu mol/L$ upstream primer, and 0.5 $\mu mol/L$ downstream primer. The upstream primer of CD23: 5'-AGAAAGCGTT-GCTGCTGTG-3' (exon 3) and downstream primer of CD23: 5'-GCCAATCCAGGAATCCTTC-3' (exon 11) were used to amplify a 695-base pair DNA product. The DNA thermal cycler (Teche PHC-3; Mandel Scientific Company Ltd., Guelph, ON, Canada) was programmed to perform a protocol as follows: 94°C for 3 minutes for 1 cycle; 94°C for 1.5 minutes, 60°C for 2 minutes, and 72°C for 3 minutes for 30 cycles; and 72°C for 7 minutes for final extension. To control for sample-to-sample variation, primers for G3PDH (upstream primer: 5'-CCATGGAGAAGGCTGGGG-3', downstream primer: 5'-CAAAGTTGTCATGGATGACC-3') were used to amplify a 193-base pair product. The PCR protocol for G3PDH was as follows: 94°C for 3 minutes for 1 cycle; 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 25 cycles; and 72°C for 7 minutes for 1 cycle. Negative controls were performed with samples lacking RNA or samples with RNA that were not reverse transcribed. The positive control was RNA from spleen cells of actively sensitized mice (presence of CD23 previously demonstrated by fluorescence-activated cell sorter). PCR products were then electrophoresed in 1.5% agarose gel in the presence of 0.5 $\mu g/mL$ ethidium bromide. The amplified DNA bands were visualized with a UV transilluminator and photographs were taken. The intensity of the DNA bands was analyzed using a densitometer with software from KODAK Digital Science 1D, GIBCO, Rockville, MD.

IgE Assay

The level of HRP-specific IgE in mouse serum was determined by passive cutaneous anaphylaxis (PCA). Sprague-Dawley rats were injected intradermally with 0.1 mL serum in duplicate dilutions from 1:1 to 1:1024. After 72 hours, rats were challenged by intravenous injection with 0.5 mL of HRP

(5 mg/mL) in 1% Evan blue. Positive reactions were evaluated as blue spots after 30 minutes. The PCA titer was recorded as the highest serum dilution showing a positive result. Heat treatment (56°C for 3 hours) abolished the reaction, indicating that the Ig was heat-labile IgE.

IL-4 Assay

Mouse spleens were meshed through a metal screen to isolate single cells. Erythrocytes in the cell mixture were lysed with ammonium chloride potassium buffer and the remaining cells were washed and incubated in 24-well plates with Concanavalin A (10 µg/mL; Sigma) for 48 hours. The supernatant was collected and tested for IL-4 by enzyme-linked immunosorbent assay (ELISA) (Biosource). The limit of detection was 5 pg/mL.

Statistics

Data were analyzed by analysis of variance. When appropriate, statistical significance was tested by two-way Student *t* tests for comparison between groups. Values are presented as mean $\pm$ SEM. A value of $P < 0.05$ was considered significant.

Results

Sensitization Enhances Epithelial Uptake of Specific Antigen

Preliminary experiments indicated that luminal antigen challenge of tissues from sensitized mice caused a rise in Isc, indicating the secretory response, which began after 2 minutes. Therefore, to examine antigen uptake in enterocytes before the hypersensitivity reaction, tissues from HRP-sensitized IL-4^{+/+} mice were removed at 2 minutes after HRP challenge and immediately fixed for electron microscopy. The number and size of HRP-containing endosomes in enterocytes were greater in tissues from HRP-sensitized mice (Figure 1C) compared with tissues from naive control mice (Figure 1A) and mice sensitized to OVA (Figure 1B). When quantified by image analysis, the area of endosomes in enterocytes of HRP-sensitized mice was 8-fold greater ($P < 0.05$) than values in naive controls and 6-fold greater than values in OVA-sensitized mice (Figure 1D). These findings suggest recognition of antigen at the level of the epithelium, potentially by cell-bound Ig.

IgE and IL-4 Are Required for Enhanced Epithelial Uptake of Antigen

To investigate if circulating factors mediate the enhanced antigen uptake in sensitized mice, HRP endosomes were evaluated in enterocytes of tissues from passively sensitized IL-4^{+/+} mice after luminal HRP challenge. Values for tissues from mice sensitized with whole

serum were compared with those in which the serum was specifically depleted of IgE or IL-4. At 2 minutes after challenge, enterocytes from mice passively sensitized with whole serum demonstrated significantly increased ($P < 0.05$) antigen uptake (greater total area of HRP endosomes) compared with values for naive control mice (Figure 2A). In fact, these values were comparable to those in actively sensitized mice. However, depletion of IgE from the serum abolished the enhanced uptake. This finding indicates that IgE is necessary for the uptake of the specific antigen into epithelial cells. Moreover, significant reduction of antigen uptake was demonstrated in mice sensitized with IL-4-neutralized serum, despite the presence of IgE, compared with values in mice injected with whole serum. Although the level of IL-4 in the serum was below the detection limit of our ELISA assay, this finding suggests that IL-4 is also involved in the enhanced transepithelial antigen uptake by a mechanism independent of its role in promoting IgE synthesis.

Sensitization Also Increases the Rate of Transepithelial Antigen Transport

In naive control mice, ~20% of enterocytes showed HRP endosomes in the apical region of cells and ~2% in the mid region, whereas no HRP was visualized in the distal regions or lamina propria at 2 minutes after HRP luminal challenge (Figure 3A). The percentage of enterocytes containing HRP endosomes in all cell regions was significantly greater in actively sensitized mice, with HRP uptake in the apical region in >70% of enterocytes, ~25% in the mid region, and ~20% in the basal region and ~5% in the lamina propria. Similarly, values were also significantly higher in passively sensitized mice compared with controls (Figure 3A). These results indicate that in both actively and passively sensitized mice, antigen was transported more rapidly across intestinal epithelial cells than in controls. Similar to results for antigen uptake, mice that were injected with IgE-depleted serum showed a slower rate of antigen transport, with the majority of HRP endosomes confined to the apical region. In addition, mice injected with IL-4-neutralized serum showed a reduced rate of transport, almost to the control values. These data provide evidence that IgE and IL-4 were involved in increasing the speed of transport of antigen across intestinal epithelial cells in sensitized mice.

Transepithelial Transport of Antigen Is Not Enhanced in Sensitized IL-4^{-/-} Mice

To further investigate the role of IL-4 in enhanced transepithelial antigen transport, HRP uptake into enterocyte endosomes was examined in IL-4^{-/-} mice ac-

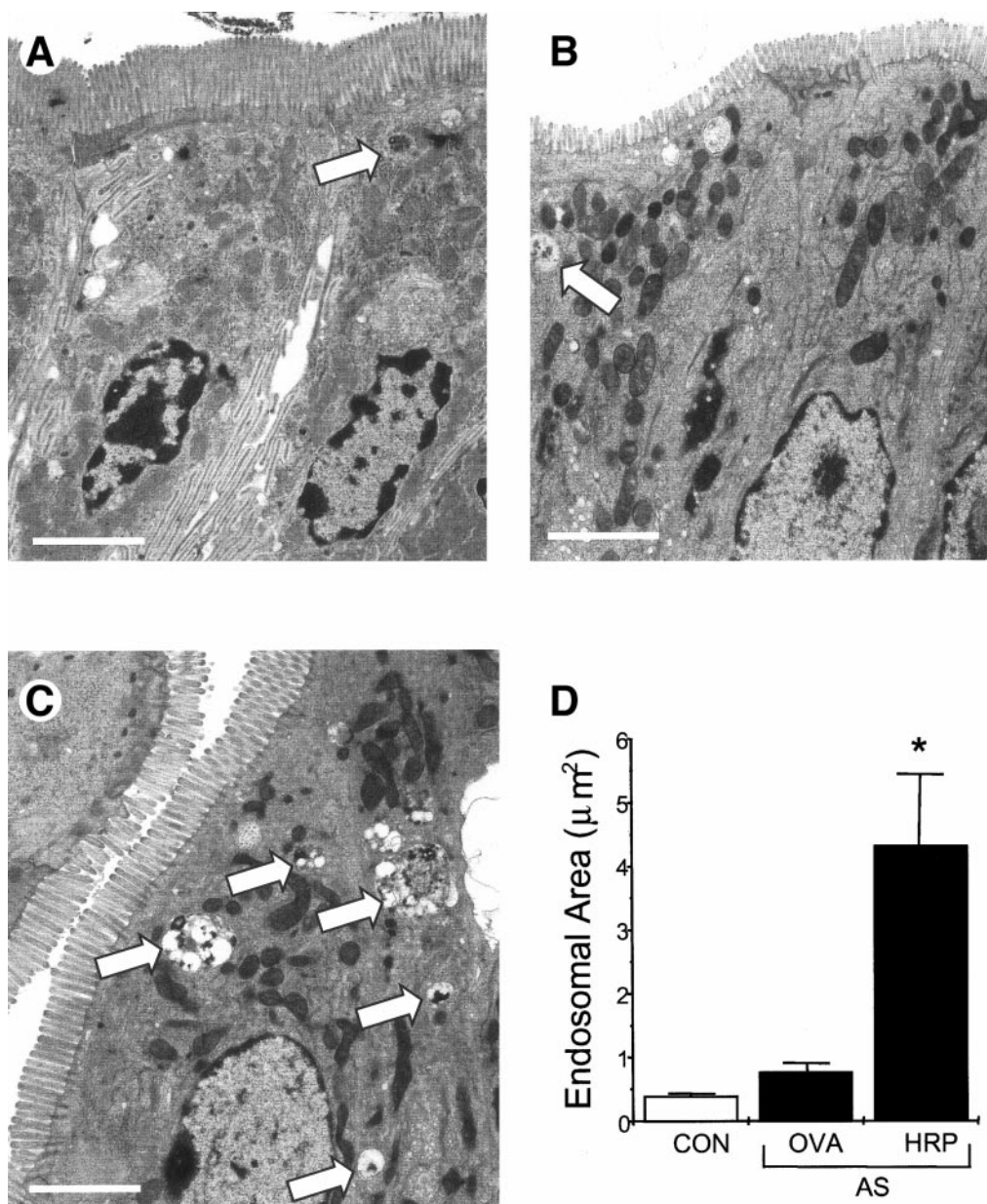


Figure 1. HRP uptake into enterocyte endosomes in control and sensitized mice. HRP (5×10^{-5} mol/L) was added to the mucosal (luminal) buffer of Ussing chambers containing jejunal tissues obtained from different groups of mice. Tissues were fixed for electron microscopy 2 minutes after HRP challenge and processed to visualize HRP uptake into endosomes. Representative photomicrographs are shown for tissues obtained from (A) naive control IL-4^{+/+} mice, (B) OVA-sensitized IL-4^{+/+} mice, (C) and HRP-sensitized IL-4^{+/+} mice. HRP endosomes are indicated by arrows (bars = 2 μ m). Original magnification 3000 $\times$. These photomicrographs are representative of those used for quantitative measurements of the endosomal area in (D). The total area of HRP endosomes was measured in electron photomicrographs by computerized image analysis within a fixed size window of 120 μ m² in the apical region (above the nucleus) of midvillus enterocytes from naive control mice (CON) and mice actively sensitized (AS) to HRP or OVA. Values represent the means $\pm$ SEM; * P < 0.05 compared with controls; n = 12 photomicrographs from 4 mice per group.

tively sensitized to HRP. The absence of IL-4 production in IL-4^{-/-} mice was confirmed by ELISA in culture media from spleen cells (undetectable for both naive and sensitized IL-4^{-/-} mice vs. 39.3 ± 1.5 pg/mL for sensitized IL-4^{+/+} mice). In contrast to the 8-fold increase in the area of HRP-containing endosomes in enterocytes of HRP actively sensitized IL-4^{+/+} mice (Figure 2A), no enhanced HRP uptake was found in enterocytes of actively sensitized IL-4^{-/-} mice compared with naive control IL-4^{-/-} mice (Figure 2B). The area of endosomes in both sensitized and nonsensitized IL-4^{-/-} mice was similar. In addition, actively sensitized IL-4^{-/-} mice did not show an enhanced rate of transport compared with control values (Figure 3B). The absence of increased trans-epithelial antigen transport may have been caused by the

lack of IgE synthesis in these mice. In fact, HRP-specific IgE was not detectable by PCA in serum from actively sensitized IL-4^{-/-} mice. Therefore, additional experiments were conducted in passively sensitized IL-4^{-/-} mice.

The area of HRP endosomes in enterocytes of passively sensitized IL-4^{-/-} mice was significantly enhanced compared with the value in control IL-4^{-/-} mice (Figure 2B). Depletion of IgE from the injected serum abolished the enhanced antigen uptake in enterocytes. In addition, the area of HRP endosomes in enterocytes of IL-4^{-/-} mice injected with IL-4-neutralized serum was not different from the value in control mice (Figure 2B). Because IL-4 is known to up-regulate the expression of the low-affinity IgE receptor, CD23, on immune cells,¹⁵⁻¹⁷

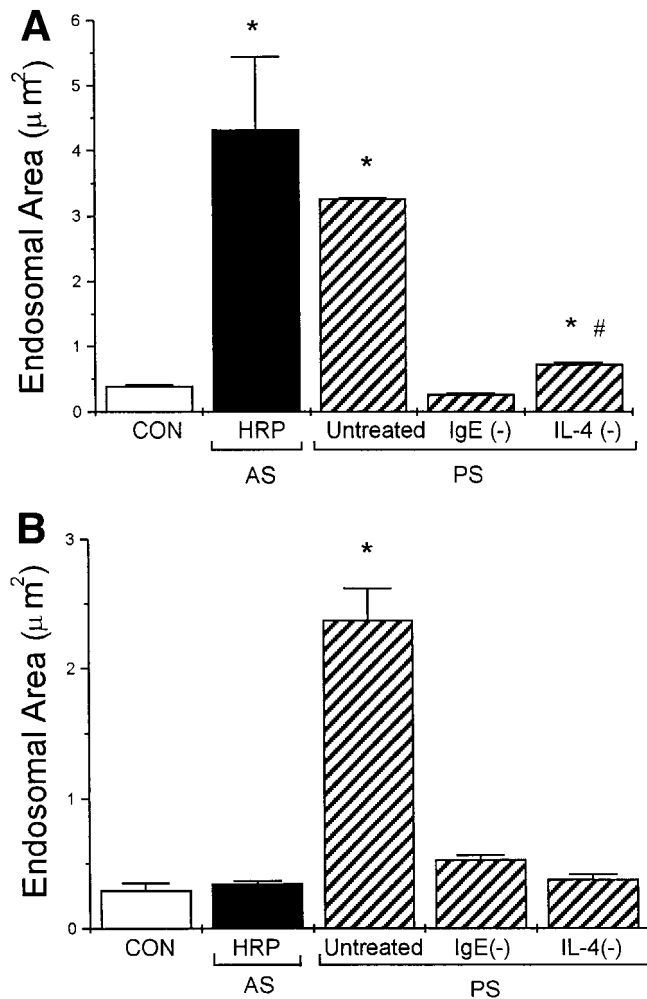


Figure 2. HRP uptake in enterocytes of actively and passively sensitized mice. Intestinal tissues from each group of mice were challenged with HRP (5×10^{-5} mol/L) from the luminal side and then fixed at 2 minutes after challenge and processed for electron microscopy. The total area of HRP endosomes in enterocytes was measured in a fixed sized apical window in electron photomicrographs and quantified in tissues obtained from (A) IL-4^{+/+} mice, either naive controls (CON), actively sensitized (AS), or passively sensitized (PS), using untreated, IgE-depleted (IgE(-)), or IL-4-neutralized serum (IL-4(-)), and (B) IL-4^{-/-} mice groups as above. Values represent the means $\pm$ SEM; * $P < 0.05$ compared with controls; # $P < 0.05$ compared with passively sensitized with untreated serum; $n = 12$ photomicrographs from 4 mice per group.

we examined the role of CD23 in enhanced antigen uptake.

Expression of the IgE Low-Affinity Receptor, CD23/FcεRII, on Intestinal Epithelial Cells Is Stimulated by Sensitization

The IgE-mediated uptake of specific antigen in enterocytes of sensitized mice suggests the involvement of an Ig receptor on intestinal epithelial cells. CD23/FcεRII, the low-affinity IgE receptor, has been shown on intestinal and airway epithelial cells of humans²⁵⁻²⁷ and

on intestinal epithelial cells in sensitized rats.¹¹ In this study, immunohistochemistry using anti-CD23 antibody²¹⁻²³ showed a low level of expression of CD23 on the apical membrane of enterocytes in nonsensitized control mice (Figure 4A). The expression of CD23 was up-regulated by sensitization, shown by increased staining on the apical membrane as well as in intracytoplasmic regions of enterocytes in actively sensitized IL-4^{+/+} mice (Figure 4B). No staining of CD23 was seen on the apical membrane of epithelial cells in both control and actively sensitized IL-4^{-/-} mice (Figure 4C and 4D) (semiquantitative analysis shown in Table 1). No stain-

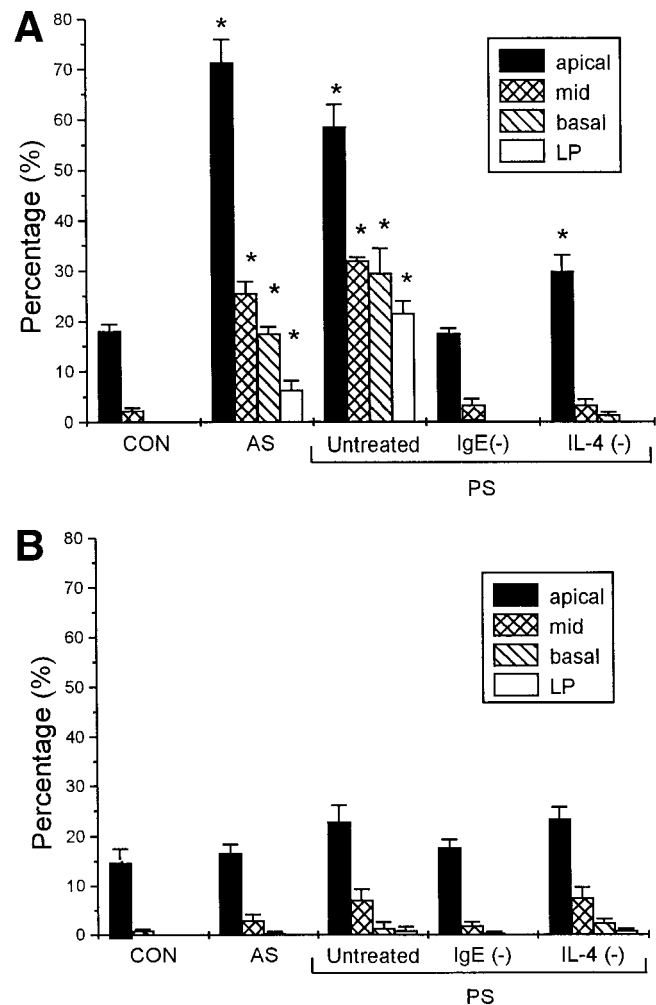


Figure 3. Rate of antigen transport across intestinal epithelial cells. Intestinal tissues from each group of mice were challenged with HRP from the luminal side and then fixed at 2 minutes after challenge and processed for electron microscopy. The percentage of enterocytes containing HRP endosomes in the apical, mid, and basal regions of cells and in the lamina propria was quantified in tissues obtained from (A) IL-4^{+/+} mice, either naive controls (CON), actively sensitized (AS), or passively sensitized (PS) using untreated, IgE-depleted (IgE(-)), or IL-4-neutralized serum (IL-4(-)), and (B) IL-4^{-/-} mice, groups as above. Values represent means $\pm$ SEM; $n = 4$ tissues examined from 4 mice per group; * $P < 0.05$ compared with controls.

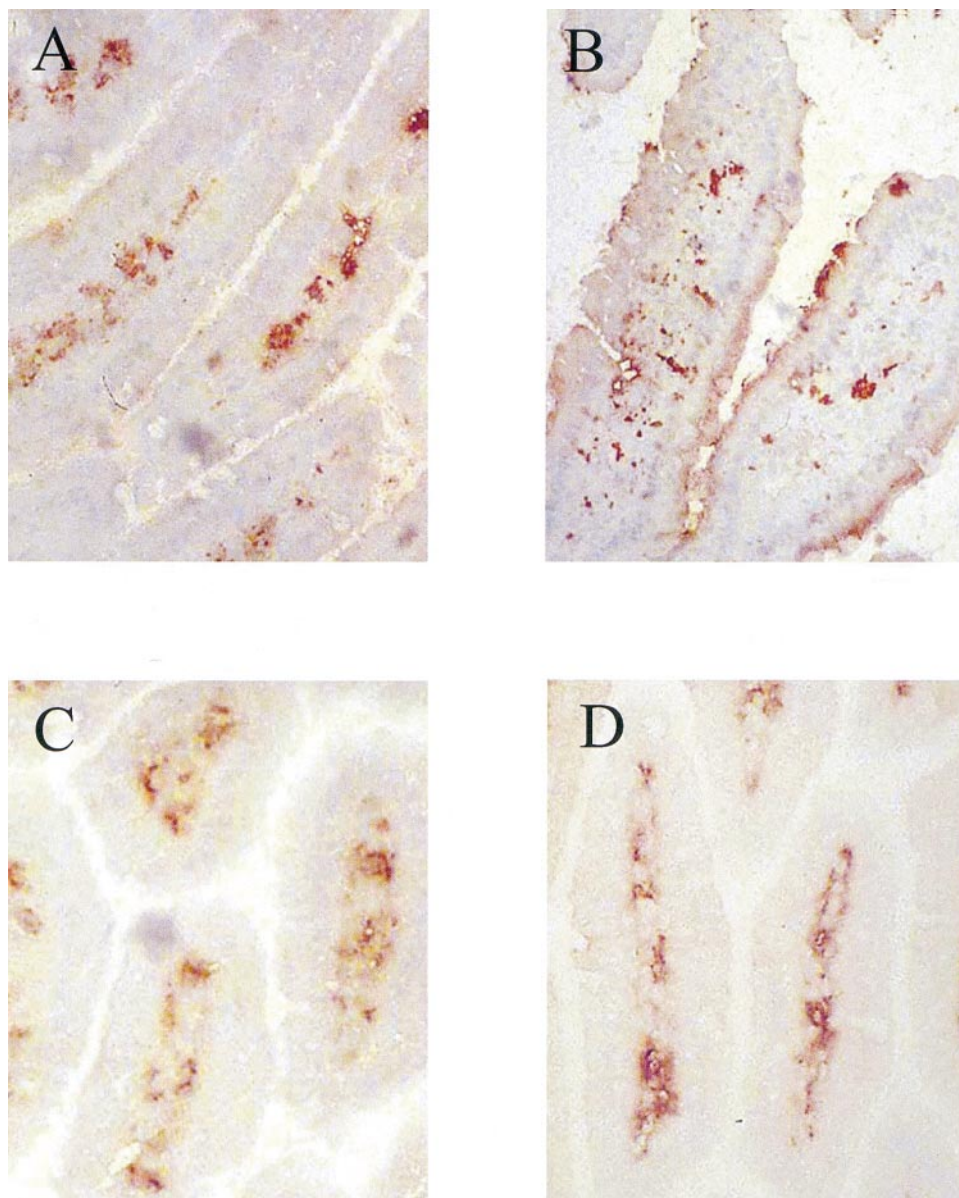


Figure 4. Expression of CD23 protein on intestinal epithelial cells. Jejunal tissues were cryofixed, sectioned, and stained with anti-CD23 using a biotin-streptavidin method. Photomicrographs shown are tissues obtained from (A) a control IL-4^{+/+} mouse, (B) an HRP-sensitized IL-4^{+/+} mouse, (C) control IL-4^{-/-} mice, and (D) HRP-sensitized IL-4^{-/-} mice. These photographs are representative of those prepared from 4 mice per group. Original magnification 200 $\times$. CD23 staining is evident on the apical membrane of enterocytes in sensitized IL-4^{+/+} mice, but not in naive controls. No staining of CD23 was seen on enterocytes of either control or sensitized IL-4^{-/-} mice. Spleen tissues from actively sensitized mice were used as a positive control, whereas negative controls included sections where the primary antibody was omitted or replaced with an isotype-matched irrelevant antibody (not shown).

Table 1. Expression of CD23 Protein on Apical Membrane of Intestinal Epithelial Cells

Mice	IL-4 ^{+/+}	IL-4 ^{-/-}
Naive control	+	-
Active sensitization	+++	-/+

NOTE. Jejunal tissues from naive or actively sensitized IL-4^{+/+} and IL-4^{-/-} mice were snap frozen, sectioned, and stained with anti-CD23 (B3B4) as primary antibody for immunohistochemistry. $n = 4$ mice per group. Spleen tissues were used as positive control. Negative controls included sections where the primary antibody was omitted or replaced with an irrelevant isotype control antibody. Semiquantitative analysis of relative staining for CD23 on epithelial cells was performed by 2 separate investigators on coded sections.

ing was seen on the surface of intestinal epithelial cells in the negative controls including sections where the primary antibody was omitted or replaced with the isotype-matched irrelevant antibody.

IL-4 Stimulates Expression of CD23 Messenger RNA in Cultured Mouse Intestinal Epithelial Cells

Reverse-transcription (RT)-PCR was performed to investigate if IL-4 induces or up-regulates CD23 transcript expression in mouse intestinal epithelial cells. Treatment of a mouse small intestinal epithelial cell line, IEC-4 cells,²⁴ with 10 ng/mL IL-4 induced increased expression of CD23 messenger RNA (mRNA), with peak expression at 8–24 hours (Figure 5).

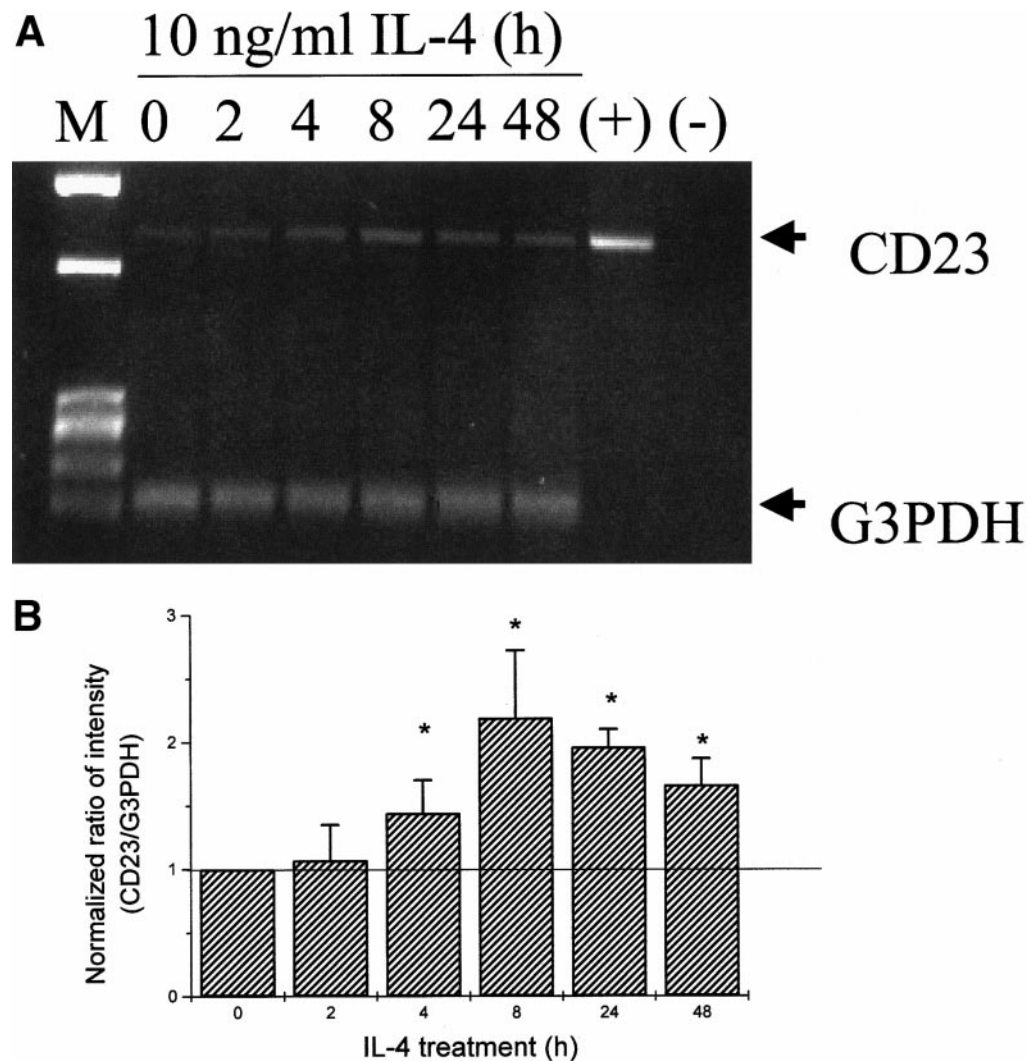


Figure 5. Effect of IL-4 on expression of CD23 mRNA by cultured mouse intestinal epithelial cells. (A) IEC-4 cells were treated with 10 ng/mL of recombinant mouse IL-4 for 0, 2, 4, 8, 24, and 48 hours. The mRNA was subjected to RT-PCR using primers for CD23 and G3PDH. PCR products were electrophoresed in a 1.5% agarose gel. Molecular weight markers (Ready load ϕ X174 RF DNA/*Hae* III fragments; GIBCO BRL) were added in the first lane. The intensity of bands was then analyzed using densitometry. (B) Normalized ratio of intensity of CD23/G3PDH at different time points after IL-4 treatment. Values represent means $\pm$ SEM; $n = 3$ individual experiments; * $P < 0.05$ compared with controls (0 h).

Enhanced Transepithelial Transport in Sensitized Mice Is Inhibited by Anti-CD23 Antibody

To investigate the role of epithelial CD23 in the enhanced transepithelial transport of antigen in intestine of sensitized mice, we used the anti-CD23 antibody added to the luminal side of the intestine in Ussing chambers 30 minutes before antigen challenge. There was a dose-dependent inhibition of antigen uptake in enterocytes by the antibody. The enhanced antigen uptake in enterocytes of sensitized mice was significantly reduced at 0.05 mg/mL of antibody ($2.7 \pm 0.1 \mu\text{m}^2$) compared with values of untreated tissues ($4.9 \pm 0.3 \mu\text{m}^2$) (Figure 6A). The rate of transepithelial transport of antigen was also diminished significantly at 0.05 mg/mL of antibody with $\sim 50\%$ of enterocytes containing endosomes in apical area, $\sim 10\%$ in the mid region, $\sim 3\%$ in the basal region, and $\sim 1\%$ in lamina propria compared with control values ($\sim 75\%$, $\sim 20\%$, $\sim 12\%$, and 6%)

(Figure 6B). Isotype control antibody up to 0.2 mg/mL did not reduce the enhanced transepithelial antigen transport (data not shown).

Transepithelial Transport of Antigen Is Not Enhanced in CD23^{-/-} Mice

Genetically targeted mice that lack CD23 were used to investigate the role of CD23 in enhanced antigen uptake. The titer of IgE in serum measured by passive cutaneous anaphylaxis was 1:256 (median value) for actively sensitized CD23^{-/-} vs. 1:128 (median value) for actively sensitized CD23^{+/+} mice. A significantly ($P < 0.05$) enhanced area of HRP endosomes in enterocytes was documented in actively sensitized CD23^{+/+} mice compared with naive controls (4-fold increase) (Figure 7A). The rate of antigen transport across enterocytes was also greater in actively sensitized mice (Figure 7B). However, in actively sensitized CD23^{-/-} mice, the area of HRP endosomes and rate of antigen transport across

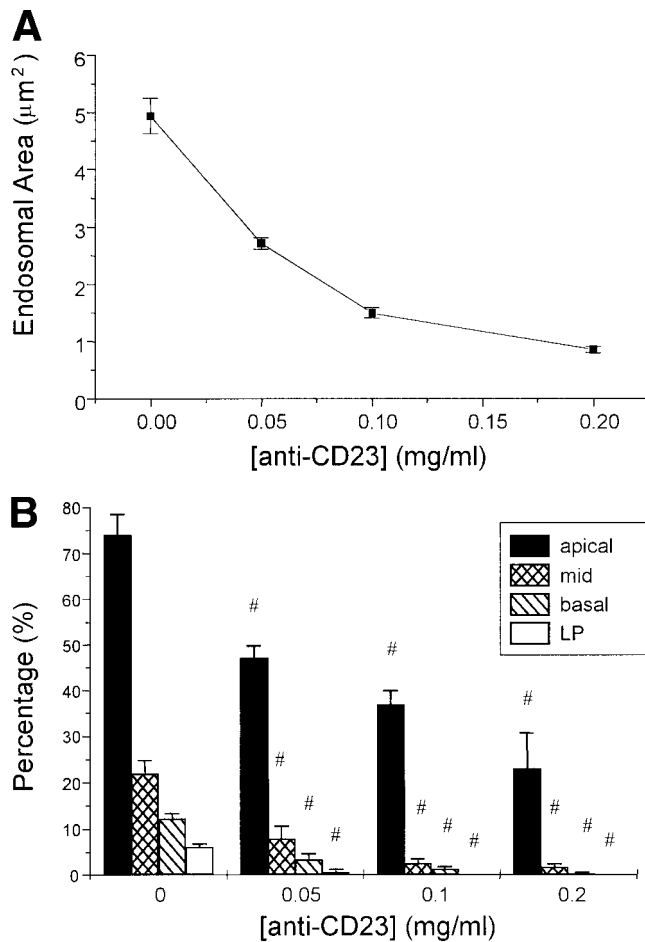


Figure 6. Effect of anti-CD23 antibody on transepithelial antigen transport. Various concentrations of anti-CD23 were added to the luminal side of Ussing chambers containing tissues from sensitized CD23^{+/+} mice 30 minutes before HRP challenge. Intestinal tissues from each group of mice were challenged with HRP (5×10^{-5} mol/L) from the luminal side and then fixed at 2 minutes after challenge and processed for electron microscopy. (A) Area of HRP endosomes in enterocytes of tissues pretreated with anti-CD23. $n = 12$ photomicrographs from 4 mice per group. (B) Percentage of enterocytes containing HRP endosomes in apical, mid, and basal regions and lamina propria (LP) in tissues pretreated with anti-CD23. $n = 4$ tissues examined from 4 mice per group. Values represent means $\pm$ SEM; # $P < 0.05$ compared with control tissues (0 mg/mL of anti-CD23).

enterocytes were comparable with values of naive CD23^{-/-} mice (Figure 7A and B).

Discussion

Our previous studies documented enhanced uptake and rapid transepithelial transport of specific antigen in jejunum of actively sensitized rats.^{8,9,11} Here, we showed that both phenomena were reproducible in another species. The use of genetically targeted mice allowed us to investigate the essential components involved in phase I antigen transport. In the current study, we provide evidence that IgE/CD23 mediates the en-

hanced transepithelial antigen transport in sensitized mouse intestine. We also show that IL-4 is a major factor regulating the mechanism by both stimulation of IgE synthesis and CD23 expression on intestinal epithelial cells.

We previously showed in sensitized rats that the quantity of antigen taken up by intestinal epithelial cells and the rate of antigen transport into the lamina propria were increased several-fold above values in naive control rats, so that at 2 minutes after luminal challenge, the antigen was already present in the lamina propria.⁸ Similarly, in HRP-sensitized mice, we found an 8-fold increase in HRP antigen uptake by enterocytes compared with naive mice or mice sensitized to an irrelevant antigen, OVA. Moreover, a greater percentage of enterocytes in HRP-sensitized mice contained HRP endosomes in the mid

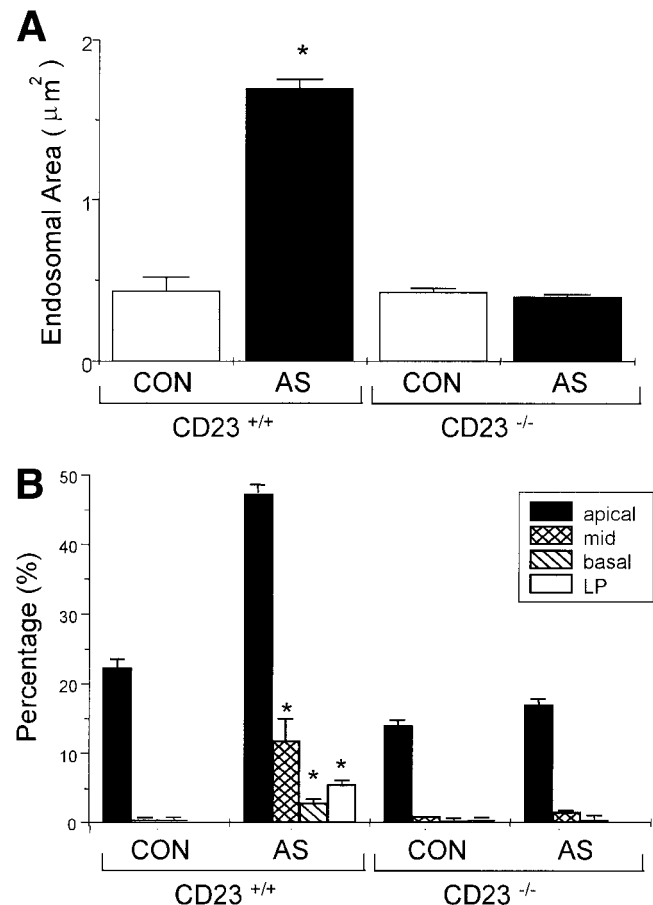


Figure 7. Transepithelial antigen transport in CD23^{+/+} and CD23^{-/-} mice. Intestinal tissues from actively sensitized (AS) and naive control mice (CON) were challenged with HRP (5×10^{-5} mol/L) from the luminal side and then fixed at 2 minutes after challenge and processed for electron microscopy. (A) Area of HRP-containing endosomes in enterocytes. $n = 12$ photomicrographs from 4 mice per group. (B) Percentage of enterocytes containing HRP endosomes in apical, mid, and basal regions and lamina propria (LP). $n = 4$ tissues examined from 4 mice per group. Values represent means $\pm$ SEM; * $P < 0.05$ compared with controls.

and basal regions of the cells and in the lamina propria compared with control and OVA-sensitized mice in which endosomes were restricted to the apical area. These results indicate that both the quantity of antigen taken up and the rate of transepithelial antigen transport were enhanced in mice sensitized to that protein, suggesting a recognition phenomenon at the level of epithelium.

Passive sensitization of mice confirmed that factors in the serum contribute to the effect of increased uptake and rapid transport of specific antigen. In contrast to whole serum, IgE-depleted serum completely abolished the increased transepithelial antigen transport, indicating that IgE antibodies mediate the enhanced luminal-to-serosal antigen transport in sensitized mouse intestine. IgE has been reported in the intestinal fluid in food allergic patients.²⁸ In rats after parasite infection, when IgE production was stimulated, the concentration of IgE in the intestinal fluid was greater than in the serum or mesenteric lymph.²⁹ In addition, radiolabeled IgE (but not IgG) translocated from the serum into the intestine and intestinal lumen after infusion of rats with IL-4.³⁰ Those findings suggested a receptor-mediated mechanism for IgE transport into the lumen. In our study, antigen uptake across the epithelium in the opposite direction, i.e., from lumen into the mucosa, required the presence of IgE. Therefore, our result also implies the involvement of an IgE receptor on the surface of epithelial cells in sensitized animals.

IL-4 regulates the synthesis of IgE in B cells by inducing germ line ϵ transcript.¹² Elevated production of IL-4 by mononuclear cells isolated from blood and intestinal mucosa of atopic individuals has been shown.^{13,14} To investigate if IL-4 is involved in the phenomenon of enhanced transport of antigen across enterocytes in sensitized animals, we used genetically targeted IL-4^{-/-} mice and IL-4-neutralized serum for passive sensitization. The absence of the enhanced uptake of HRP into enterocyte endosomes in actively sensitized IL-4^{-/-} mice suggests that IL-4 is involved in increased transepithelial antigen uptake in sensitized animals. However, as shown previously³¹ and confirmed by us, IL-4^{-/-} mice do not produce IgE, which may be one factor accounting for the lack of effect. Importantly, IL-4^{+/+} mice passively sensitized with IL-4-neutralized serum also showed diminished enterocyte uptake of HRP antigen compared with mice sensitized with untreated serum, although the value was greater than that in naive controls. In contrast, IL-4 neutralization completely abolished enhanced uptake of antigen in enterocytes of IL-4^{-/-} mice. (Previously published studies indicate cross-reactivity of IL-4 for rat and mouse receptors on cells other than B cells because of the

unique conformation of the receptor [IL-4R α and IL-13R α subunits] on these cells.)³²⁻³⁵ Because IgE remained in the IL-4-neutralized serum, these findings suggest that IL-4 is involved in the enhanced antigen uptake by inducing changes in addition to stimulation of IgE production. In addition, passive sensitization using IL-4-neutralized serum abolished the rapid transport of antigen across enterocytes in IL-4^{+/+} mice in comparison to those injected with untreated serum, providing evidence that IL-4 was involved not only in enhancement of antigen uptake, but also in rapid transepithelial antigen transport.

One of the important functions of IL-4 is up-regulation of the expression of IgE receptors on immune cells.¹⁵⁻¹⁸ The high affinity IgE receptor, Fc ϵ RI, is mainly expressed on mast cells and basophils.³⁶ In atopic diseases, the binding of allergens to IgE causes cross-linking of Fc ϵ RI on the mast cell surface, which leads to cell activation and degranulation. However, this receptor has not been reported on enterocytes. The low affinity IgE receptor, CD23, has been identified on epithelial cells. Bronchial epithelial cells from brushings of the airway of asthmatic patients, but not control subjects, showed positive staining for CD23.²⁷ Human cultured keratinocytes expressed CD23 after IL-4 treatment.³⁷ Importantly, expression of CD23 was demonstrated on apical membrane of enterocytes in biopsy specimens from normal individuals and seemed to be greater in individuals with food allergy and other enteropathies.²⁵ In our recent study,¹¹ we showed that sensitization of rats induced the expression of CD23 on the apical membrane of enterocytes, as detected by both immunohistochemistry and immunogold labeling. In addition, the number of CD23 labels on the cell surface was decreased after antigen challenge, and internalization of CD23 was shown by the presence of CD23 labels on the membrane of endosomes containing antigen.¹¹

In this study of sensitized mice, we identified the expression of CD23 protein on the apical membrane of intestinal epithelial cells by immunohistochemistry, with up-regulated expression in actively sensitized IL-4^{+/+} mice compared with naive controls. In contrast, low to negligible expression of CD23 was seen on the surface of enterocytes in actively sensitized IL-4^{-/-} animals. The absence of CD23 expression on the surface of enterocytes paralleled the lack of enhanced transepithelial antigen uptake. The increased expression of epithelial CD23 induced by sensitization in IL-4^{+/+} mice, but not IL-4^{-/-} mice, is likely caused by activation of the IL-4 enhancer element on the CD23 gene.³⁸ IL-4 is produced in excess in lymphocytes isolated from intestinal mucosa

in allergic individuals,¹⁴ leading to a high local concentration of IL-4 in the lamina propria that may activate the CD23 gene in intestinal epithelial cells. We also showed that IL-4 up-regulates CD23 mRNA expression in a mouse intestinal epithelial cell line, IEC-4, after 8–24 hours. Because of the difficulties of isolating a pure population of epithelial cells from mouse intestine for RT-PCR studies, we did not measure the level of CD23 mRNA in enterocytes of sensitized and control mice. Taken together, our results in IL-4^{+/+} and IL-4^{-/-} mice and cultured cells suggest that IL-4 affects transepithelial antigen transport by stimulating both IgE production and CD23 expression on enterocytes.

The role of CD23 in facilitating enhanced antigen transport by intestinal epithelial cells in sensitized mice was substantiated by dose-dependent antibody inhibition. The addition of the well-characterized anti-CD23 antibody, B3B4,^{21–23} to the luminal surface of intestine 30 minutes before antigen challenge, reduced the enhanced antigen uptake in enterocytes and significantly decreased the rate of transport across the epithelium. In contrast, the isotype control antibody did not inhibit enhanced antigen transport. CD23 has been previously reported to mediate antigen uptake and focusing in B cells, and this effect was inhibited by pretreatment with B3B4.²³ B3B4 has been shown to compete with IgE for binding to CD23 with a higher affinity,²¹ therefore, we suggest that this antibody displaces IgE from its receptor on the epithelial cells that leads to reduced endocytosis of antigen.

Studies in sensitized CD23^{-/-} mice provided further evidence of the involvement of CD23 in enhanced antigen uptake and transport. No enhanced uptake and transport of antigen was seen in enterocytes of sensitized CD23^{-/-} mice compared with sensitized CD23^{+/+} mice or naive controls. CD23^{-/-} mice have been reported to display normal lymphocytic differentiation and were able to mount normal antibody responses, including IgE responses, upon immunization and parasitic infection.^{19,39–41} In agreement with others,^{19,41} sensitized CD23^{-/-} mice exhibited higher or comparable specific IgE levels compared with sensitized CD23^{+/+}. Despite the presence of a high titer of HRP-specific IgE in the serum of actively sensitized CD23^{-/-} mice, there was no enhanced uptake of HRP in intestinal epithelial cells upon luminal antigen challenge. Together with the anti-CD23 inhibition, this result strongly supports the role of epithelial CD23 in binding IgE and mediating enhanced antigen uptake and rapid antigen transport in sensitized intestine.

Normally, proteins internalized in endosomes are transported to the supranuclear region of enterocytes

where the vesicles fuse with lysosomes. Lysosomal enzymes degrade these macromolecules in an acidic compartment.⁴² However, transepithelial transport of IgA and IgG has been shown under physiologic conditions. Polymeric Ig receptor (receptor for IgA) and FcRn (receptor for IgG) have been identified on the basal and apical surface of intestinal epithelial cells.^{43,44} It has been suggested that receptor-mediated transport of protein within endosomes across intestinal epithelial cells may bypass degradative lysosomal pathways.^{43,44} Whether CD23 enhances transepithelial antigen transport of intact antigens and IgE by a similar mechanism in hypersensitivity requires further investigation.

In summary, our study has documented enhanced transport of specific antigen across intestinal epithelial cells after luminal challenge in both actively and passively sensitized mice. Our results provide evidence that IgE/CD23 mediates the enhanced transepithelial antigen transport in sensitized intestine because both IgE depletion and anti-CD23 antibody reduced epithelial uptake of antigen. Moreover, we showed that IL-4 plays a major role in the mechanism of phase I–enhanced transepithelial antigen transport by regulating both IgE synthesis and expression of CD23 on intestinal epithelial cells. Finally, we suggest that antigen binding to IgE/CD23 may bypass the intracellular degradative pathway for proteins resulting in large quantities of antigen penetrating the epithelial barrier. An understanding of the mechanism involved in enhanced transepithelial antigen transport may allow the development of novel therapeutic interventions to reduce adverse anaphylactic reactions in food allergy.

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Received February 13, 2001. Accepted May 16, 2001.

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Supported by a grant from the Canadian Institutes of Health Research (to M.H.P.) and a Rx&D Scholarship (to L.C.H.Y.).