

CD23-Mediated IgE Transport Across Human Intestinal Epithelium: Inhibition by Blocking Sites of Translation or Binding

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Background & Aims: In previous studies in rodent models of food allergy, we identified that sensitization induces expression of CD23 on intestinal epithelial cells and results in enhanced IgE-dependent transepithelial antigen uptake; further studies in CD23^{-/-} mice provided evidence that CD23 is involved in protected transport of antigen into the body. Little information exists in humans on receptor-mediated immunoglobulin (Ig)E transport across epithelia. The present study was designed to examine expression of CD23 by human epithelial cells, determine its isoform and regulation by interleukin (IL) 4, and identify the role of CD23 in transepithelial IgE transport. **Methods:** Epithelial expression of CD23 was studied in cell lines, ileal biopsy specimens, and explanted fetal intestine. Bidirectional transport of IgE was measured across filter-grown cells, either normal cells or those transfected with antisense CD23 oligonucleotides, or in the presence of blocking antibody. **Results:** Expression of the low-affinity IgE receptor was demonstrated in cultured epithelial cells as well as in situ cells in human intestine. CD23b was the isoform expressed by HT29, T84, and Caco-2 cells. IL-4 up-regulated the expression of epithelial CD23. IgE was transported in both the basal-to-apical direction and the apical-to-basal direction across filter-grown epithelial cells, a process that was inhibited by transfection of cells with CD23 antisense oligonucleotides or pretreatment with nonspecific IgE or anti-CD23 antibody. **Conclusions:** These findings provide evidence that CD23 encodes a functional IgE receptor on human intestinal epithelial cells and that this epithelial receptor is likely to play an important role in food allergies.

The prevalence of allergic diseases in general, and food allergy in particular, has increased consistently over the past 10 years. True food allergy (immune-mediated hypersensitivity) affects 2%–5% of adults and an even greater proportion (~8%) of children.¹ Following the ingestion of a specific food antigen, sensitized individuals develop local gastrointestinal symptoms such as diarrhea, nausea, and vomiting and often respiratory and cutane-

ous symptoms. Common allergens include cow's milk, egg, nuts, shellfish, and others.^{1,2} In severe cases such as an allergy to peanuts, systemic anaphylaxis can occur and be life threatening. There are few effective treatment strategies apart from avoidance of the offending foods. However, particularly in young children, restricted diets may compromise nutrition. Therefore, it is important to understand the mechanisms underlying food allergic reactions to develop novel therapeutic strategies.

Immunoglobulin (Ig)E is the immunoglobulin isotype involved in immediate hypersensitivity allergic reactions. Circulating IgE antibodies are present in allergic individuals.^{1–3} IgE has also been measured in intestinal fluid and feces of food allergic patients.^{4–6} In contrast, it is below the limit of detection in both blood and luminal contents of nonreactive humans. It is not clear how individuals become sensitized to foods because proteins encountered in the gastrointestinal tract normally result in oral tolerance (actively down-regulated immune responses).⁷ However, atopy can be inherited, and IgE has been measured even in cord blood of neonates.⁸ Interleukin (IL) 4 is the key T-lymphocyte-helper, type 2 (Th2) cytokine involved in switching antibody responses to IgE.⁹ In addition, IL-4 induces the expression of IgE receptors on mast cells and other cell types.^{10–12} The hypersensitivity reaction is initiated by antigen cross-linking of IgE bound to high-affinity receptors on the surface of mast cells. This, in turn, triggers mast cell degranulation and release of inflammatory mediators (including histamine, lipid mediators, proteases, and oth-

Abbreviations used in this paper: EBV, Epstein Barr virus; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FcRn, neonatal immunoglobulin receptor; HRP, horseradish peroxidase; Ig, immunoglobulin; IL, interleukin; OVA, ovalbumin; pIgR, polyimmunoglobulin receptor; RT-PCR, reverse-transcriptase polymerase chain reaction; SCID, severe combined immunodeficiency; Th, T helper.

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ers) that act on nearby cells to alter physiology.¹³ However, in the gastrointestinal tract, mast cells are located beneath the epithelium, which should restrict the uptake of intact protein antigens.

The epithelium is composed of a single-celled layer of epithelial cells joined together by tight junctions. It performs the critical function of nutrient absorption while, at the same time, excluding antigenic macromolecules and microbes. There is evidence that the intestinal epithelial barrier is abnormal in humans with allergies and animal models of food allergy. Children with milk allergy have increased uptake of milk protein.¹⁴ Increased permeability has also been described in patients with eczema and asthma.^{15,16} Our studies in sensitized rats demonstrated that the sensitizing protein is preferentially taken up across the gut epithelium.^{17,18} We showed that the initial route of antigen transport is transcellular via endosomes, resulting in increased amount and rate of antigen uptake; following activation of mast cells, the paracellular pathway is recruited, resulting in an overall nonspecific reduction in barrier function.¹⁹ Our recent studies identified that CD23 (the low-affinity receptor for IgE) is expressed on the cell membrane of epithelial cells in sensitized rats and mice.^{20,21} Evidence that CD23 is involved in enhanced antigen transport was obtained from experiments using anti-CD23 antibodies and in CD23^{-/-} mice,²⁰⁻²² in which both antigen transport and hypersensitivity reaction were inhibited. Taken together, our findings suggest that the enhanced transepithelial antigen transport in sensitized rodents is mediated by binding to IgE-CD23 on the apical surface of epithelial cells.

CD23, the low-affinity receptor for IgE (Fc ϵ RII), is a type II transmembrane glycoprotein with an extracellular domain (containing the IgE-binding site) belonging to the C-type animal-lectin family.²³⁻²⁵ Two isoforms (CD23a and CD23b, which differ in the N-terminal cytoplasmic portions of the molecule) of human CD23 have been cloned. CD23a is constitutively expressed on B cells and possibly eosinophils,^{25,26} whereas CD23b is inducible by IL-4 on various immune cell types, including B cells, monocytes, macrophages, neutrophils, and others.²⁷⁻³⁰ In addition, CD23 protein has been identified on human keratinocytes³¹ and intestinal epithelial cells,^{32,33} but its isoform and function have not been completely elucidated.

The present study was designed to examine the expression of CD23 by human epithelial cells, its regulation by IL-4, and its role in IgE transport. We documented epithelial expression of this IgE receptor (the CD23b isoform) in 3 cultured cell lines as well as in situ cells in human intestine. Because IgE is produced by B

(plasma) cells located in the intestinal lamina propria or other body sites, we examined IgE transport in the basal-to-apical direction as well as the apical-to-basal direction across filter-grown epithelial cells. We determined that IL-4 up-regulates the expression of CD23 on epithelial cells. Bidirectional transepithelial transport of IgE was enhanced following IL-4 treatment. Finally, anti-CD23 antibody or transfection of cells with CD23 antisense oligonucleotides inhibited transepithelial IgE transport. These findings provide evidence that CD23b encodes a functional IgE receptor on human intestinal epithelial cells that is likely to play an important role in food allergic conditions.

Materials and Methods

Antibodies and Chemicals

The anti-CD23 antibody used in this study was monoclonal mouse anti-human CD23, clone Tu1 (NovoCastra Laboratories Ltd, Newcastle Upon Tyne, United Kingdom). Human IgE anti-NP (against 4-hydroxy-3-nitrophenylacetyl), and the κ IgE (κ IgE) fragment as well as NP-ovalbumin (OVA) were from Serotec Inc. (Oxford, United Kingdom). A rabbit polyclonal antibody against the ϵ chain of human IgE (DAKO Diagnostics, Mississauga, ON, Canada) was used to identify IgE. Recombinant human IL-4 was a product of R&D Systems Inc (Minneapolis, MN). Culture media and reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA). IgG3 and the protease inhibitor cocktail were from Sigma-Aldrich (St. Louis, MO). The F(ab')₂ fragment of IgG was from Jackson ImmunoResearch Laboratories (Westgrove, PA).

Cell Lines and Culture Methodology

Cell lines were from stock supplies maintained at McMaster University. The human epithelial cell lines used in this study were T84, HT29, and Caco-2. T84 cells were cultured in Dulbecco's modified Eagle medium (DMEM): F-12 media (1:1), supplemented with 10% fetal bovine serum (FBS), 0.15% NaHCO₃, 1.2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin. HT29 cells were cultured in DMEM medium with 5% FBS, 0.0375% sodium bicarbonate, 0.2 mmol/L L-glutamine, and penicillin/streptomycin. Caco-2 cells were cultured in DMEM, supplemented with 10% FBS, 1% nonessential amino acids, and penicillin/streptomycin. The cells were split every 5 days, and media were changed every 2 or 3 days. Human THP-1 monocytes and EBV-transformed B lymphocytes were cultivated in RPMI 1640 media, with (for THP-1 cells) 10% FBS, 35 mmol/L HEPES, 100 U/mL penicillin, and 100 μ g/mL streptomycin or (for B cells) 100 mmol/L HEPES, 1.6 mmol/L L-glutamine, 5% FBS as well as penicillin/streptomycin. All cells were incubated in an atmosphere of 5% CO₂ at 37°C.

Intestinal Biopsy Specimens and Xenografts

Approval for these studies was obtained from the research ethics board and the animal research ethics board at McMaster University. Human intestinal biopsy specimens (distal ileum) were obtained from 3 patients undergoing workup for possible inflammatory bowel disease (subsequently identified as normal). Human fetal small intestine was acquired within 14 to 15 weeks of gestation. Intestinal tissues ($n = 6$) were transplanted subcutaneously into severe combined immunodeficient (SCID) mice and allowed to develop for an additional period of 14 weeks as described.³⁴ Mice were injected intraperitoneally (IP) with 0.3 mL IL-4 (10 $\mu\text{g/mL}$) or saline 48 and 24 hours before they were killed. Xenograft tissues were removed and placed on ice. Tissue samples and biopsy specimens were placed in optimal cutting temperature, frozen, and processed for immunohistochemistry.

CD23 Messenger RNA Expression by Reverse-Transcriptase Polymerase Chain Reaction

The published sequence of human CD23 messenger RNA (mRNA) indicates 2 isoforms of CD23, type *a* and type *b*.^{23,25,27} Based on this information, we designed primers: for CD23*a*: 5'-ATG GAG GAA GGT CAA TAT T-3' and 5'-CTC CCA TCC ACC CAG ATA AA-3'; and for CD23*b*: 5'-GGA CGC AAT AGA GTC AGA G-3' and 5'-CCT GGC TTG GAG GAT TCA T-3'. The cDNA for CD23*b* and CD23*a* were cloned and authenticated by DNA sequencing. GAPDH primers were as follows: 5'-CCA TGG AGA AGG CTG GGG-3' and 5'-CAA AGT TGT CAT GGA TGA CC-3'.

Total RNA from epithelial cell lines, monocytes, or Epstein Barr virus (EBV)-transformed B cells was extracted using QIAGEN RN easy kit (Qiagen Inc., Mississauga, ON, Canada). Cells were disrupted in RNA lysis buffer, and then 1 volume of 70% ethanol was added to the homogenized lysate. The sample solution was then applied to an RNeasy minispin column and centrifuged for 30 seconds at 13,000 rpm. RNA collected in the column was washed with buffers RW1 and RPE successively. The total RNA extracted was eluted in RNase-free water, divided into small aliquots, and stored at -80°C . Reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out using GeneAmp RNA PCR Core kit (Applied Biosystems, Branchburg, NJ): 2 μg total RNA was added to reagent mixture for reverse transcription (including dNTP, MuLV reverse transcriptase, Oligo d(T)₁₆) and reacted for 15 minutes at 42°C . PCR was executed in a PCR master mixture with AmpliTaq DNA polymerase and primers for 30 cycles. Each cycle consisted of the following: denaturing 1 minute at 95°C , annealing 1 minute at 60°C , and synthesizing 1 minute at 72°C , finally extended at 72°C for 7 minutes. The PCR products were loaded onto a 3.0% agarose gel, including 50% lower melting point agarose, for electrophoresis. The image of PCR fragments was photographed, and the integrated

intensity was determined by scanning densitometry using GAPDH as an internal control for the total amount of RNA added. Negative controls were performed with samples lacking RNA or with RNA that was not reverse transcribed. The software for whole-band analysis was from BioImage (Ann Arbor, MI).

Antisense Transfection

Three antisense oligodeoxynucleotides were designed around the protein translation start site: the upstream antisense (5'-CCT GGC TTG GAG GAT TCA T-3'), middle antisense (5'-TGG AGG ATT CAT TAT GCT AA-3'), and downstream antisense (5'-CAT TAT GCT AAA TTC TGC TT-3'). A nonsense oligodeoxynucleotide (5'-TTC GTC TTA AAT CGT ATT AC-3') was also used; its design was neither complementary to any part of the CD23 gene nor to any known human sequence.

The sense, antisense, and nonsense oligodeoxynucleotides were transfected into HT29 cells using the lipid-based OligofectAMINE reagent (Invitrogen Life Technologies). The transfection mixture was prepared according to the manufacturer's instructions with minor modifications. In brief, oligonucleotides were diluted in serum-free cell culture medium to 300 nmol/L and incubated for 15 minutes. Next, the transfection reagent OligofectAMINE was incubated with the serum-free culture medium for 10 minutes. Finally, both were mixed together (transfection mixture) and incubated for a further 15-minute period at room temperature. Before transfection, the cells were washed with serum-free medium. The transfection mixture was then added to the cells and incubated for 4 hours in 37°C , CO_2 atmosphere. After this interval, the culture medium with 3X normal serum was added to the cells (without removing the transfection mixture) for a further 48-hour incubation.

CD23 Protein Expression by Western Blot Analysis

Cells were washed 3 times with ice-cold PBS (pH 7.2) containing a cocktail of protease inhibitors (Sigma). Cells were harvested (by scraping) into 500 μL ice-cold lysis buffer: 100 mmol/L NaCl, 10 mmol/L Tris-Cl, 2 mmol/L EDTA, 1.8% Triton X-100, and protease inhibitors (pH 7.8). Total protein in the supernatant of the cell lysate was measured by Bio-Rad kit (Bio-Rad Laboratories, Mississauga, ON, Canada). Protein (60 μg for each sample) was loaded onto a 10% polyacrylamide gel, allowed to migrate, and analyzed by SDS-PAGE. The protein bands were transferred electrophoretically onto a nitrocellulose membrane and incubated with anti-human CD23 antibody (1:500) for 48 hours at 4°C , with shaking. After 3 washes with PBS, horseradish peroxidase (HRP)-conjugated secondary antibody (1:40,000) was added and incubated for 2 hours. An enhanced chemiluminescence method was used to detect the target CD23 protein using a LumiGlo substrate kit (KP Laboratories, Gaithersburg, MD) and x-ray film. The integrated intensity for the protein bands was determined by scanning densitometry.

CD23 Protein Expression by Immunohistochemistry

CD23 protein expression was examined in cultured epithelial cells, intestinal biopsy specimens, and xenograft tissues. Epithelial cells were grown on coverslips for 3–5 days. The cells were fixed with 50% acetone buffered in PBS for 15 minutes. Biopsy specimens were obtained and immediately frozen at -80°C . Cryostat sections were cut at a thickness of 8 μm and fixed in cold acetone for 15 minutes. After washing, the endogenous peroxidase activity was quenched by 3% H_2O_2 for 15 minutes and further treated with blocking buffer (0.1 mol/L Tris-Cl, 0.15 mol/L NaCl, 0.5% blocking reagent, pH 7.5) for 30 minutes. Cells or biopsy specimens were incubated with anti-CD23 primary antibody (1:100) for 2 hours and further incubated in biotinylated secondary antibody (1:1000) for 30 minutes. The signal was amplified using streptavidin-HRP and tyramide fluorescein (TSA kit; NEN Life Science Products Inc, Boston, MA). Xenograft tissue was removed from the SCID mice, frozen, and treated similarly, except that the sections were cut at a thickness of 6 μm , and the dilutions for primary and secondary antibodies were 1:50 and 1:2000 (Cy-5-conjugated anti-mouse antibody), respectively. The nuclei in the tissue were counterstained with propidium iodide for 10 minutes. Negative controls involved substitution of a control antibody (IgG₃) for the primary antibody. The images were observed in a confocal microscope and analyzed using LSM510 software.

IgE Binding and Uptake

HT29 cells were cultured in 24-well plates for 3 days. Human IgE, (0.04 $\mu\text{mol/L}$) diluted in a IgE-binding buffer (10 mmol/L Tris-HCl, 140 mmol/L NaCl, 2 mmol/L CaCl_2 , 1 mmol/L MgCl_2 , 1.8 g/L glucose or mannitol, pH 7.35),³⁵ was added to the cells and incubated for 70 minutes at 4°C or 37°C . The cells were prepared for immunohistochemistry as indicated above. To determine whether IgE had been internalized, cells were washed 3 times with ice-cold PBS, scraped from the wells in lysis buffer (including protease inhibitors), and protein used for Western blotting analysis. IgE was recognized by a horseradish peroxidase (HRP)-conjugated rabbit polyclonal antibody against the ϵ chain of human IgE, and the enhanced chemiluminescence technique was then employed to detect the labeled immunoglobulin. Studies were conducted in the presence of anti-CD23 antibody (vs nonspecific control IgG₃ antibody) to determine the effect of blocking CD23 on IgE binding.

IgE Transport

IgE transport across monolayer cultured epithelial cells was carried out using HT29 cells grown on 0.4 μm , pore-size Transwell filters (Corning Inc., New York, NY). Cells (0.8×10^6) were seeded onto the filter either normally or on the reverse side,³⁶ and the cells were cultured for 7 days until confluent (resistance at least 300 Ω/cm^2). Human IgE (0.4 $\mu\text{mol/L}$ in IgE-binding buffer) was added to either the basal or

apical compartment of the Transwells. After incubation at 37°C for specific periods of time, media samples from the opposite compartment were collected and stored at 4°C for IgE analysis by Western blot or ELISA. Studies were conducted in the presence of anti-CD23 antibody (vs nonspecific control IgG₃ antibody) to determine the effect of blocking CD23 on IgE transport.

HT29 cells were chosen for these studies because preliminary experiments indicated that IL-4 did not alter the permeability of HT29 monolayers for macromolecules, whereas T84 cell monolayers demonstrated increased permeability after treatment with IL-4.³⁷ To confirm this, transport of a nonspecific protein, horseradish peroxidase (HRP; added at 10 $\mu\text{mol/L}$) was measured for comparison. The concentration of intact HRP was determined by assaying enzyme activity using a modified Worthington method as described.³⁷ Briefly, 150 μL sample was added to 800 μL phosphate buffer containing 0.003% H_2O_2 and 80 $\mu\text{g/mL}$ o-dianisidine (Sigma Chemical Co, St Louis, MO). Enzyme activity was determined from the rate of increase in OD at 460 nm.

Enzyme-Linked Immunosorbant Assay for IgE

A sandwich ELISA method was employed to measure IgE. A rabbit polyclonal antibody against the ϵ chain of the human IgE (10 $\mu\text{g/mL}$) (DAKO Diagnostics) was used to coat 96-well plates then washed 3 times with PBS. Samples were added and incubated with the antibody overnight at 4°C (with shaking). After 3 washes with PBS, a monoclonal mouse anti-human IgE (1:1000) (DAKO Diagnostics) antibody was introduced to detect the captured IgE. After 3 washes, the HRP-conjugated secondary antibody was added to the plate for 1-hour incubation. The color reaction was performed using OPD tablets (DAKO Diagnostics), which were prepared according to the manufacturer's instruction. The reaction was stopped by the addition of 0.5 mol/L H_2SO_4 . The color absorbance was read at 490 nm in a plate reader (Bio-Tec Inst. Inc., Winooski, VT).

Inhibition of IgE Binding and Transport by Nonspecific IgE

IL-4-treated HT29 cells were incubated for 1 hour at 4°C with various concentrations of either F(ab')_2 fragment of IgG (used as a negative control) or κIgE (full structure of the $\text{Fc}\epsilon$ heavy chain without the light chain; used as nonspecific IgE to compete with specific IgE anti-NP for binding to NP-OVA). IgE anti-NP at 50 $\mu\text{g/mL}$ was then added to the cells for 90 minutes. Subsequently, the cells were washed 3 times with ice-cold PBS, containing protease inhibitors and then lysed. The IgE anti-NP was measured by a specific ELISA. Briefly, the wells of a 96-well plate were coated overnight with NP-OVA (1 $\mu\text{mol/L}$), washed, and blocked with 1% BSA and 5% sucrose in PBS for 1 hour. Supernatants from the lysed cells were added to the wells at suitable dilutions and incubated for 2 hours. After 3 washes, HRP-conjugated rabbit anti-human ϵ chain antibody was added and incubated for 1

hour. After 3 additional washes, HRP substrate was added to the wells. After 20 minutes, the reaction was stopped, and the concentration was measured at an optical density of 450 nm.

For transport studies, IL-4-treated HT29 cell monolayers grown on the filter supports were employed. F(ab')₂ or κ IgE (0.4 μ mol/L) fragments were incubated with the monolayer for 30 minutes at 37°C. IgE anti-NP (0.4 μ mol/L) was then added. After 2 hours, media in the opposite compartment was collected on ice, and the transported IgE anti-NP was measured as described above.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Differences between groups were tested by analysis of variance, with post hoc analysis by Newman-Keuls test or Student *t* test when appropriate. *P* values less than .05 were accepted as significant.

Results

Human Intestinal Epithelial Cells Express CD23 mRNA

CD23b mRNA was constitutively expressed in all 3 intestinal epithelial cell lines, T84, HT29, and Caco-2, as well as in THP-1 and EBV B cells (Figure 1, upper panel), whereas CD23a was expressed only in EBV B cells (Figure 1, lower panel). IL-4 increased CD23b mRNA expression in HT29 cells in a time-dependent manner (Figure 2A). Compared with naive control cells, greater expression of CD23 mRNA occurred after 8 hours of incubation with 10 ng/mL IL-4. Expression was still elevated after 16 and 24 hours. CD23 mRNA expression increased in a concentration-dependent man-

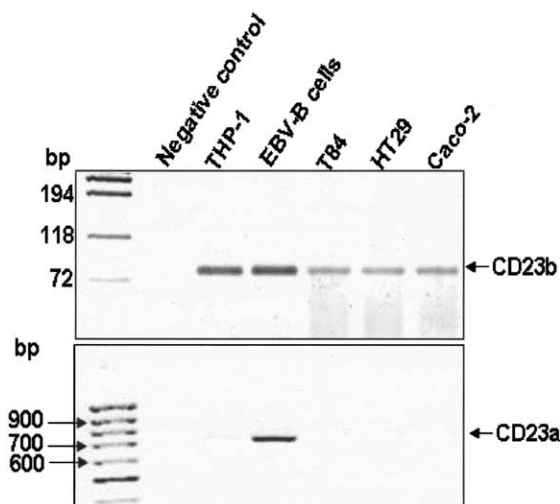


Figure 1. Constitutive expression of CD23 (a/b) mRNA. Extracted total RNA from THP-1, EBV B cells, and intestinal epithelial cells (HT29, T84, Caco-2) was subjected to RT-PCR using primers for CD23b (upper panel) and CD23a (lower panel). CD23b was constitutively expressed in THP-1, EBV B-cells, HT29, T84, and Caco-2 cells, and CD23a was constitutively expressed only in EBV B cells.

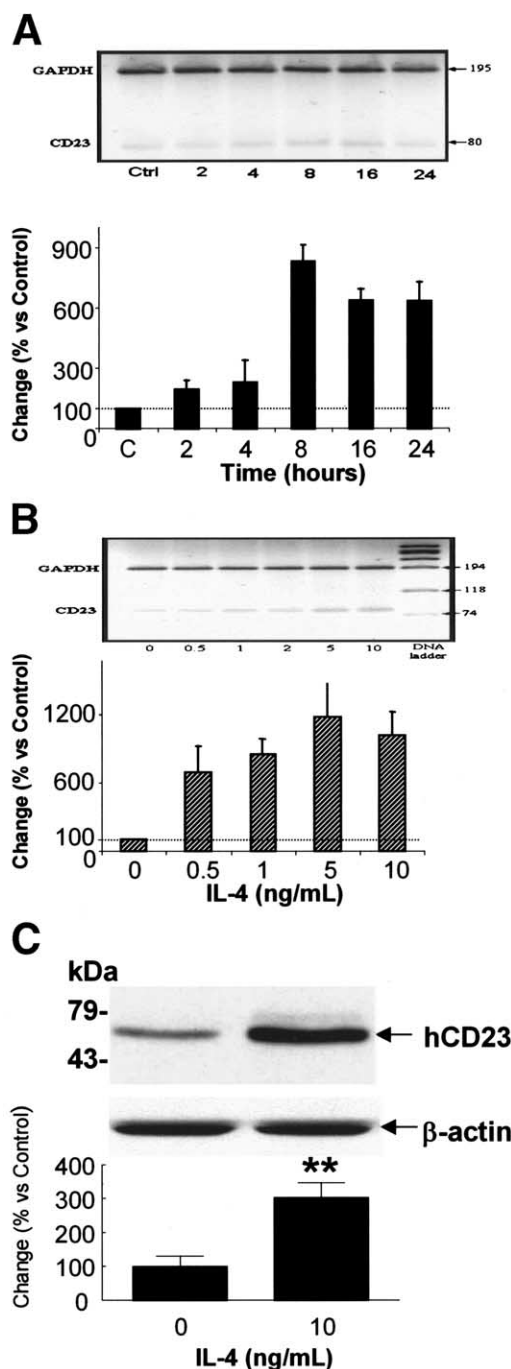


Figure 2. Effect of IL-4 on CD23 expression in epithelial cells. (A) HT29 cells were incubated with 10 ng/mL IL-4 for different times. The PCR products were separated by electrophoresis and photographed, and the integrated intensity was determined by scanning densitometry using GAPDH as an internal control. Bars indicate the percentage increase of CD23 mRNA expression compared with zero time (0) controls (mean $\pm$ SE; *n* = 4 individual experiments). (B) HT29 cells were incubated with IL-4 for 8 hours at different concentrations. Bars indicate the percentage increase of CD23 mRNA expression compared with no IL-4 (0) controls (mean $\pm$ SE; *n* = 4 individual experiments). (C) CD23 protein expression indicated by Western blots in cells incubated with or without IL-4 at 10 ng/mL for 24 hours. Bars indicate the percentage increase of band intensity compared with no IL-4 (0) controls (mean $\pm$ SE; *n* = 5 individual experiments; ***P* < .01 compared with control). Representative gels are shown for each condition.

ner when HT29 cells were incubated for 8 hours with increasing concentrations of IL-4 (Figure 2B). Maximal expression was obtained with 5–10 ng/mL of IL-4. A similar expression pattern was observed for T84 and Caco-2 intestinal epithelial cells (not shown).

Human Intestinal Epithelial Cells Express CD23 Protein

Figure 2C shows that CD23 protein expression in HT29 cells, measured by Western blots, was increased 3-fold by culturing cells for 24 hours with 10 ng/mL of IL-4. Immunofluorescence staining revealed that cells of all 3 human epithelial cell lines, HT29, Caco-2, and

T84, expressed CD23 protein (Figure 3A; d,f,h). CD23 protein was also expressed by epithelial cells in ileal biopsy specimens (Figure 3A, b), mainly on the apical membrane but also on the basolateral cell membrane. Immunofluorescent staining of intestinal xenografts implanted into SCID mice illustrated that implanted tissue did not express CD23 protein constitutively (Figure 3B, upper right panel). In contrast, xenografts implanted in mice injected with IL-4 expressed epithelial CD23 (Figure 3B, lower right panel). Here, the staining appeared to be intense on the apical membrane and within the apical cytoplasm. The specificity of the secondary antibody was confirmed by substituting an IgG3 control

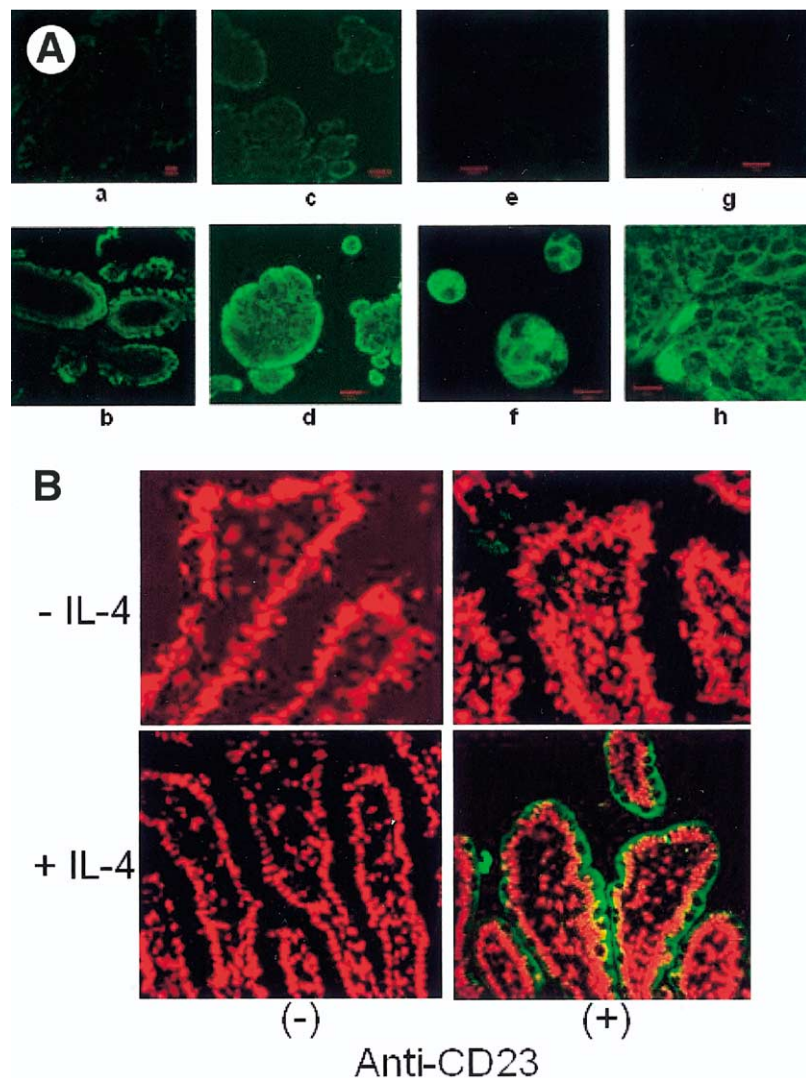


Figure 3. Immunofluorescence staining of CD23 on intestinal epithelial cells. (A) The upper panels show sections treated with IgG3 isotype control antibody, whereas the lower panels show sections treated with anti-CD23 antibody, prior to secondary antibody: (a,b) human ileal biopsy; (c,d) HT29 cells; (e,f) Caco-2 cells; (g,h) T84 cells. These images are representative of those obtained in 3 separate studies (bar = 20 μ m). (B) In these sections of human fetal intestinal xenografts, the epithelial cell nuclei are stained red and CD23 protein stained green. The lower panels are sections of human intestine from mice injected with IL-4, whereas the sections in the upper panels are of human intestine from control untreated mice. Negative control sections are shown in the left panels.

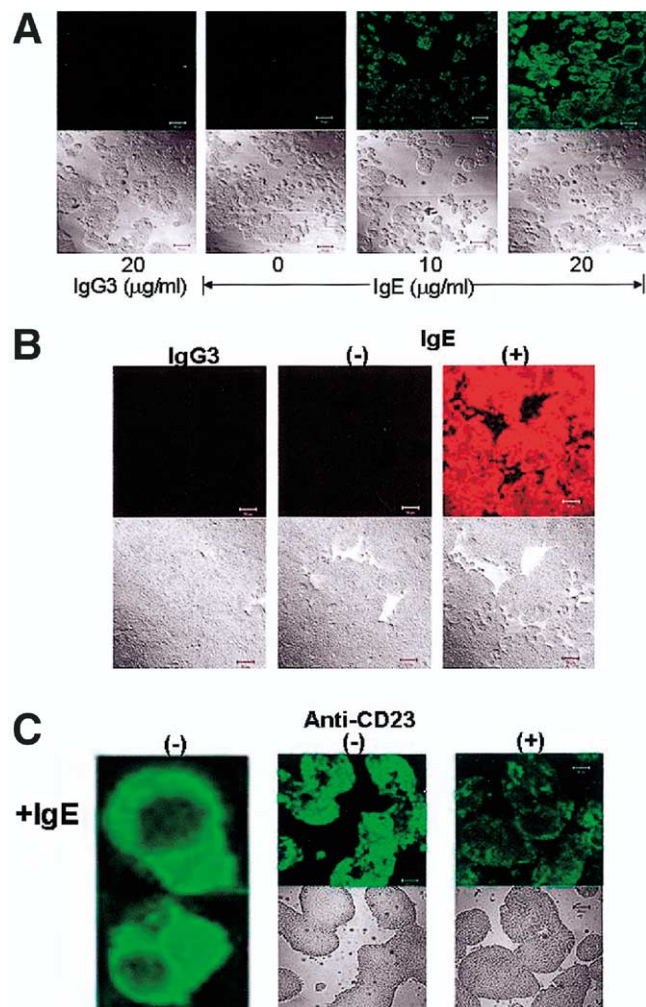


Figure 4. IgE binding to and uptake into epithelial cells. (A) Binding. The upper panels are confocal images illustrating IgE binding to HT29 cells (grown with 10 ng/mL IL-4) after incubation at 4°C for 70 minutes. IgE, identified by staining with anti-IgE antibody (green), is visualized on the surface of the cells. IgG3 was used for the negative control. The lower panels are phase contrast images of the cells (bars = 50 µm). (B) Uptake. The upper panels are laser confocal images illustrating IgE uptake into HT29 cells after incubation at 37°C for 70 minutes. In this case, IgE stained red is seen within cells. IgG3 was used for the negative control. The lower panels are phase contrast images of the cells (bars = 50 µm). (C) The right panel shows the effect of exposing HT29 cells to anti-CD23 for 30 minutes before adding IgE. CD23 antibody inhibited IgE uptake (bar = 20 µm). The left panel shows a higher magnification of untreated individual cells with internalized IgE and unstained nucleus. The antibodies were used at 190 µg/mL.

antibody for the primary antibody (Figure 3A; a,c,e,g and 3B, left panels).

Epithelial CD23 Is Responsible for the Binding, Uptake, and Transport of IgE

Binding of IgE to HT29 epithelial cells was examined after incubating IgE with HT29 cells at 4°C. The IgE bound mainly to the outer edges of nonconfluent

cell clusters (Figure 4A), with more intense staining at the higher concentration of IgE. When the cells were incubated at 37°C, IgE appeared to be located within the cells, as shown in Figure 4B and 4C (left panel). This was confirmed by Western blots of cell lysates (not shown).

Figure 5 illustrates the effect of IL-4 treatment of filter grown cells on IgE transport. There was very little transport of IgE across untreated cell monolayers. Significantly greater transport of IgE was demonstrated when cells were cultured with IL-4 for 24 hours, as illustrated by the Western blot prepared of media samples obtained from the compartment opposite to where the IgE was added (Figure 5A, upper panels). Enhanced uptake occurred regardless of whether the IgE was added to the apical or basal compartment. To determine the specificity of CD23 for IgE, cells treated with IL-4 were

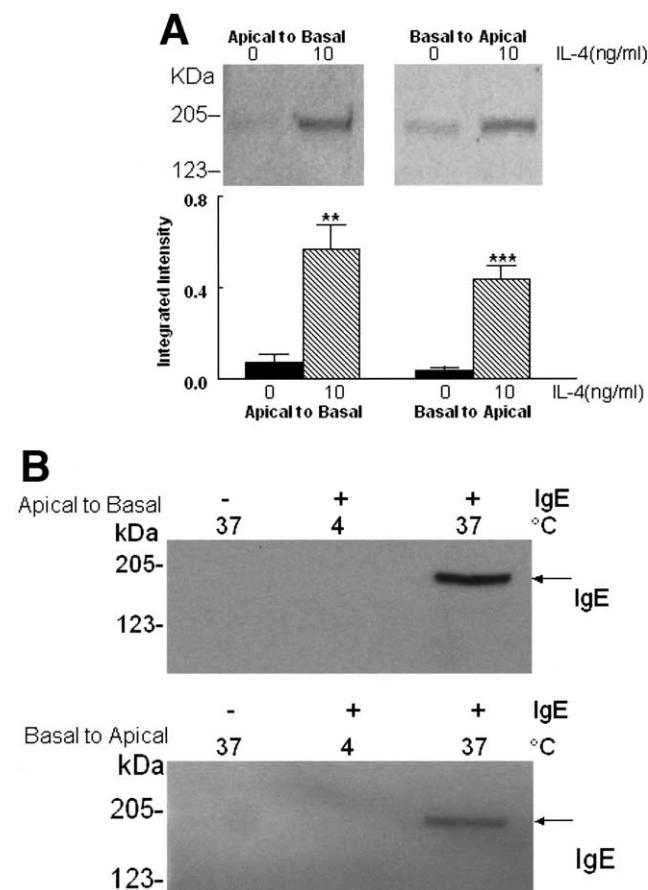


Figure 5. Trans epithelial transport of IgE. (A) HT29 cells were grown (for 8 days until confluent with IL-4 included for the final 24 hours) on either side of Transwell filters. IgE was then added to either the apical or basal compartment, and samples were obtained from the opposite compartment after 2 hours. IgE was indicated by Western blots as shown in the upper panels. The lower panels illustrate the integrated intensity of the bands determined by densitometry (mean $\pm$ SE; n = 4–6 individual experiments; ** P < .01, *** P < .005 compared with controls). (B) Experiments were conducted as in panel A, except that the effect of incubation at 4°C was compared with incubation at 37°C. Cold temperature completely inhibited trans epithelial transport of IgE.

exposed simultaneously to apical IgE and IgG3. There was no difference in IgE transported in the presence or absence of IgG3, indicating lack of competition for the receptor. Metabolic activity was required for IgE transport because cells incubated at 4°C did not demonstrate transport in either direction (Figure 5B). These findings were confirmed by ELISA: a significant increase ($P < .005$) in the amount of IgE transported across the monolayers treated with IL-4 compared with control monolayers: $20.3 \pm 0.6 (\times 10^{-4})\%$ vs $10.6 \pm 1.9 (\times 10^{-4})\%$ recovery of added IgE. In contrast, there was no difference in the transport of the nonspecific protein HRP across IL-4-treated monolayers vs untreated monolayers: $13.0\% \pm 2.3\%$ vs $16.1\% \pm 3.0\% (\times 10^{-3})$, respectively.

CD23-Mediated IgE Uptake and Transepithelial Transport Are Inhibited by anti-CD23 and Antisense Transfection

Pretreatment of HT29 cells with anti-CD23 antibody concentration dependently inhibited IgE uptake into epithelial cells, as illustrated by Western blots of lysed cells (Figure 6A). Preincubation of cells with IgG3 (190 $\mu\text{g}/\text{mL}$) for 30 minutes had no effect on IgE uptake into cells (fold increase from control values in cells exposed to IgE alone vs IgG3 plus IgE: 1.8 ± 0.2 vs 2.3 ± 0.2 , respectively) (Figure 6B). In addition, compared with monolayers treated with IgG3, anti-CD23 antibody significantly ($P < .01$) decreased the amount of IgE transported across cell monolayers in either direction (Figure 6C).

Antisense oligonucleotides were also used to examine the effect of reduced expression of CD23 on IgE transport. In 4 experiments, the upstream antisense decreased the IL-4 enhanced expression of CD23 mRNA in HT29 cells by a mean of $\sim 50\%$, whereas the middle and downstream antisense oligonucleotides were less effective (Figure 7A). Cells transfected with the upstream antisense oligonucleotide significantly decreased the amount of IgE transported across the epithelial monolayers (Figure 7B). In contrast, the nonsense oligonucleotide had no effect on IgE transport.

CD23-Mediated IgE Uptake and Transepithelial Transport Are Inhibited by Nonspecific IgE

Specificity for IgE binding by CD23 was determined by examining competition for the receptor by 2 forms of IgE. We used κIgE to block the cell sites and measured subsequent binding and transport of IgE anti-NP. Figure 8A shows that only IgE anti-NP was detected by the ELISA (wells coated with NP-OVA) in a

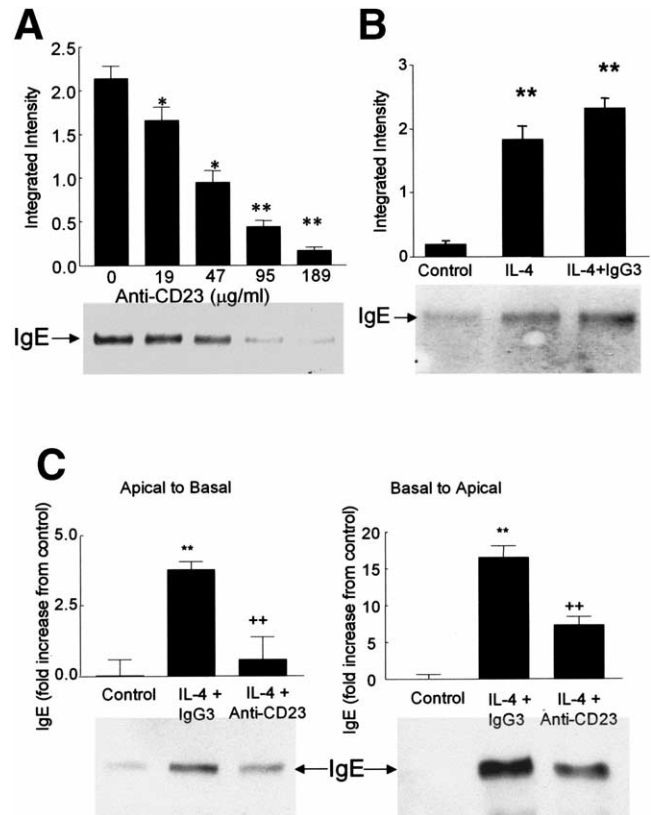


Figure 6. Inhibition of IgE uptake or transport by anti-CD23 antibody. (A) Plate-grown HT29 cells (grown with IL-4 for 24 hours) were incubated with anti-CD23 antibody at different concentrations for 30 minutes at 37°C prior to adding IgE. After 70 minutes of incubation with IgE, the cells were washed with PBS and lysed. IgE was determined by Western blotting (lower panel). Anti-CD23 antibody concentration-dependently inhibited IgE uptake as illustrated by the integrated intensity values obtained by densitometry. (B) IgG3 (190 $\mu\text{g}/\text{mL}$) was substituted for anti-CD23 and had no effect on IgE uptake. (C) Filter-grown HT29 cells were incubated with anti-CD23 or IgG3 antibody (190 $\mu\text{g}/\text{mL}$) for 30 minutes at 37°C prior to adding IgE (both added to either the apical or basal compartment). After 2 hours, buffer was collected from the opposite compartment. Western blots of the buffers demonstrated inhibition of IgE transport in the apical-to-basal direction and the basal-to-apical compartment by anti-CD23. Values indicate mean $\pm$ SE; $n = 4$ individual experiments; * $P < .05$, ** $P < .01$ compared with control, ++ $P < .01$ compared with IgG3. (Control cells were grown without IL-4.)

concentration-dependent manner, whereas the nonspecific κIgE in the concentration range 0.39–100 ng/mL did not bind to the wells. Pretreatment of epithelial cells with the control F(ab')_2 IgG fragment did not block the binding of IgE anti-NP to the IL-4-treated HT29 cells (Figure 8B). However, pretreatment of the cells with κIgE inhibited ($\sim 50\%$) the binding of specific IgE anti-NP in a concentration-dependent and saturable manner. Similarly, Figure 8C shows that F(ab')_2 at 0.4 $\mu\text{mol}/\text{L}$ had no effect on the transport of IgE anti-NP, whereas κIgE at the same concentration induced significant inhibition ($P < .01$ compared with control). Neither F(ab')_2 nor κIgE alone interfered with the assay.

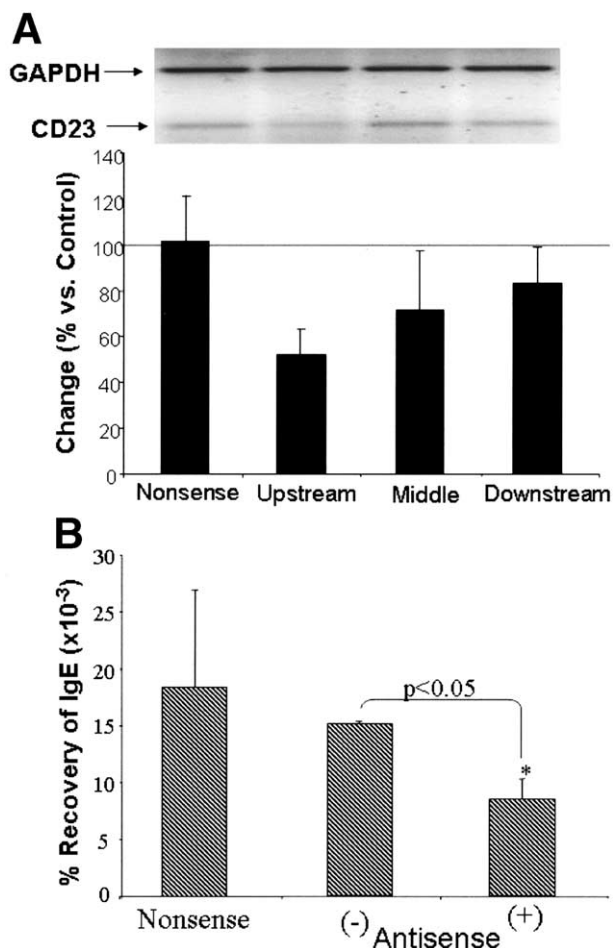


Figure 7. Effect of transfection of cells with antisense oligonucleotides on CD23 mRNA expression and IgE transport. (A) CD23 mRNA expression is shown in HT29 cells incubated with either nonsense, upstream, middle, or downstream antisense oligonucleotides for CD23 mRNA (added at the same time as 10 ng/mL IL-4). Bars indicate the percentage change of CD23 mRNA expression compared with controls at 100%, determined by integrated intensity of bands (mean $\pm$ SE; $n = 4$ individual experiments). A representative gel is shown. (B) HT29 cells were grown on Transwell filters with IL-4 and upstream antisense oligonucleotides prior to addition of IgE to the apical compartment. The bars indicate the percentage recovery of the IgE in the basal compartment (mean $\pm$ SE; $n = 4$ individual experiments; * $P < .05$). The upstream antisense oligonucleotides decreased the amount of IgE recovered in the basal buffer compared with the control, whereas the nonsense oligonucleotide had no effect.

Discussion

To our knowledge, this is the first study to identify that CD23 expressed in human enterocytes is a receptor involved in the binding and bidirectional transport of IgE across polarized intestinal epithelial monolayers. Cultured epithelial cells, as well as those in human intestine, demonstrated protein expression of CD23, with the mRNA isoform in cultured cells being CD23b. In addition, we determined that expression of CD23 (both message and protein) by enterocytes is regulated by

IL-4. Our studies indicate that CD23-mediated trans-epithelial IgE transport is a physiologic process responsible for the rapid uptake and delivery of this immunoglobulin across the intestinal epithelial barrier.

In our previous studies in rodent models of food allergy,^{20,21} we identified that sensitization or IL-4 treatment induces expression of CD23 on intestinal epithelial cells, stimulates production of IgE, and enhances trans-epithelial antigen uptake. The data supported the hypothesis that the accelerated transport of antigen is me-

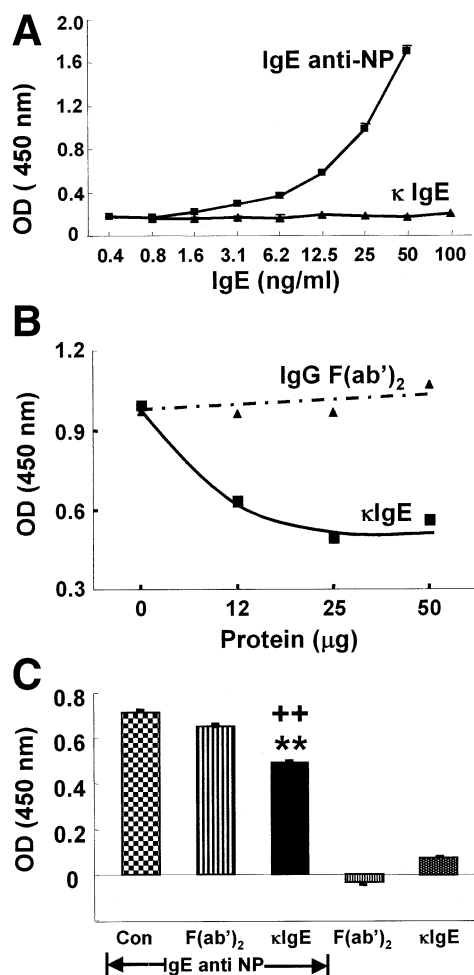


Figure 8. Effect of blocking CD23 with nonspecific IgE on IgE binding and transepithelial transport. (A) ELISA (wells coated with NP-OVA) showing that only IgE anti-NP was detected in a concentration-dependent manner, whereas the nonspecific κ IgE did not bind to the wells. (B) IL-4-treated HT29 epithelial cells were preincubated with either a control F(ab')₂ IgG fragment or the κ IgE fragment before exposing the cells to IgE anti-NP (all at 4°C). After washing, the cells were lysed to recover IgE anti-NP, which was measured by the ELISA. κ IgE inhibited the binding of IgE in a concentration-dependent saturable manner. (C) Transepithelial transport (37°C) of IgE anti-NP was measured across epithelial cell monolayers treated with either F(ab')₂ or κ IgE at 0.4 μ mol/L. F(ab')₂ had no effect on the transport of IgE anti-NP, whereas κ IgE at the same concentration induced significant inhibition. Values indicate mean $\pm$ SE; $n = 3$ individual experiments; ** $P < .01$ compared with control (Con); ++ $P < .01$ compared with F(ab')₂.

diated by binding to antigen-specific IgE attached to CD23 on the apical cell surface and that subsequent endocytosis protected the antigen from degradation during transcytosis. This implied that the IgE is first transported from the body (either from the circulation or intestinal mucosal cells) across the epithelium toward the lumen. To examine bidirectional transepithelial transport of IgE in this study, we chose an in vitro human cell culture model because human intestinal epithelial cells are known to form a tight monolayer. In addition, although CD23 expression was previously reported on human enterocytes,^{32,33} the isoform was not indicated nor was the functional role of this protein described. Therefore, the present studies were designed to identify the CD23 isoform expressed by human intestinal epithelial cells, to define its regulation by IL-4, and to determine the role of CD23 in transepithelial IgE transport.

Here, we showed that 3 different cultured intestinal epithelial cell lines express CD23*b* mRNA constitutively, but not CD23*a*, in contrast to EBV B cells, which express only CD23*a*. Epithelial cell CD23*b* message appeared to be time and concentration dependently up-regulated by IL-4. CD23 protein was similarly identified in cultured epithelial cells in which expression was increased 3-fold by IL-4. To determine whether CD23 is expressed by nontransformed human intestinal epithelial cells, we examined small intestinal biopsies. Enterocytes in "normal" ileal biopsy specimens (from humans with no identified intestinal disease) showed staining mainly on the brush border apical membrane, with some staining also evident on the basolateral membrane. However, using biopsy specimens, we were not able to determine the effect of IL-4 because the cells may have been exposed previously to this cytokine or other stimulatory factors in vivo. Therefore, we examined CD23 protein expression in human fetal intestinal xenografts by immunohistochemistry. CD23 protein was not evident on epithelial cells of intestinal xenografts implanted into SCID mice unless the mice were previously injected with IL-4. A previous study³⁸ reported expression of mRNA for both CD23*a* and CD23*b* in fetal intestinal tissue during the first and second trimester; however, the specific cells expressing the receptors were not examined and may have been immune cells. Our study does not rule out expression of isoforms other than CD23*b* in intact tissues. Clearly, in situ hybridization studies are necessary for conclusive evidence regarding the isoforms of CD23 expressed by specific cell types in vivo. The absence of CD23 protein expression by fetal enterocytes in our study may indicate lack of constitutive expression, but it may also be due to the absence of a required growth

factor in mice that is present in cell culture media and in vivo.

Several previous studies have described expression of CD23 by human cells, with both isoforms, *a* and *b*, being reported, although in different cell types. The *a* isoform is constitutively expressed in B cells, whereas IL-4 induces the expression of isoform *b* in B cells, monocytes, eosinophils, and keratinocytes.^{27,39} The amino acid sequences of the CD23 proteins encoded by the different isoforms CD23*a* and *b* differ only in their 6/7 N-terminal residues, a region that corresponds to the cytoplasmic domain,²⁷ suggesting that this region regulates divergent intracellular trafficking and/or signalling pathways. In B cells, CD23 mediates antigen uptake and focusing³⁸ with different patterns, depending on the isoform.

Several findings in the current studies indicate that human epithelial CD23 is a functional receptor for IgE. IgE binding to CD23 on the apical membrane of HT29 cells took place at 4°C, and internalization occurred only when the temperature was raised to 37°C. Growing enterocytes on both sides of filter supports allowed us to examine bidirectional transepithelial transport of IgE. IgE was transported across high-resistance epithelial monolayers in both directions, basal-to-apical and apical-to-basal, with recovery values being quite similar. IgE transport across untreated cell monolayers was very small compared with those incubated with IL-4 for 24 hours. IL-4 did not affect the transport of the bystander protein HRP, suggesting that IL-4 influences IgE transport by stimulating expression of CD23 rather than by inducing nonspecific increases in permeability of the epithelial monolayer. Like internalization of IgE into single cells, transepithelial transport was dependent on physiologic temperature.

In contrast to our findings, Yokota et al⁴⁰ reported that murine macrophages transfected with CD23*b* failed to internalize IgE alone, although IFN- γ -treated cells phagocytosed IgE-coated erythrocytes. In the study of Karagiannis et al,⁴¹ B cells incubated with antigen-free IgE did not take up the immunoglobulin after 2 hours, whereas immune complexes were efficiently internalized. We have previously reported in studies involving rodent gut tissues and epithelial cells²² that intestinal epithelial cells express unique splice variants of CD23*b*. Those results together with findings from ongoing rodent studies suggest that different splice variants transport IgE vs immune complexes, whereas the classical form of CD23*b* does not endocytose IgE. Although we have not yet documented the expression of similar splice variants in human enterocytes, it is possible that polar epithelial cells in general express CD23 proteins, which differ in form and function from those expressed by nonpolar

immune cells. In addition, in the studies described in this study, we have employed an IgE-binding buffer,³⁵ which facilitates IgE binding and uptake. Some of these factors may explain the differences between our results and those in symmetrical cell types. However, because all the critical studies have yet to be carried out, we cannot draw final conclusions regarding the role of CD23 isoforms and subtypes.

IgE transport was inhibited by pretreatment of cells with anti-CD23 antibody. Compared with the isotype control antibody, which had no effect, anti-CD23 concentration dependently reduced the amount of IgE internalizing into the enterocytes, and transepithelial transport was reduced by 50%–80%. Transfection of cells with the upstream antisense oligonucleotides, which reduced expression of CD23 by ~50%, also reduced IgE transport by ~50%. In contrast, transfection of cells with nonsense oligonucleotides had no significant effect. To confirm the specificity of CD23 for IgE, we conducted additional experiments using an IgE fragment that has the Fc ϵ portion but no light chain. We showed that this nonantigen-specific IgE fragment was able to inhibit binding of IgE to epithelial cells in a concentration-dependent and saturable manner and to reduce significantly its uptake across the cell monolayer. The maximum inhibition of binding was ~50% and of transport was ~30%. This may be due to the fact that, unlike an antibody, κ IgE can be displaced from the receptor by IgE-anti-DNP, and, therefore, both types of IgE were bound and transported simultaneously. These results, taken together with the temperature sensitivity of IgE transport, provide evidence to support the concept that transepithelial transport of IgE is mediated by the CD23 receptor expressed on the cell surface of enterocytes. However, our results do not rule out the possibility that another mechanism or receptor may also be involved in the transport of IgE across gut epithelium.

Receptors for other immunoglobulins have been identified on human enterocytes. These include the polyimmunoglobulin receptor (pIgR) for IgA and the neonatal receptor FcRn for IgG (actually demonstrated in both neonatal and adult intestine). Serosal-to-luminal transport of IgA mediated by pIgR has been known for many years, with uptake of immune complexes being described in the opposite direction.^{42–44} Apical uptake of IgG bound to FcRn and its transcytosis within endosomes was described^{45–47} with IgG being subsequently detected free in the paracellular spaces, suggesting that it is released from cells. In our studies, IgE was clearly released after transport, although we have not determined the relative quantity of free IgE vs that which remains associated with its receptor. The exact trafficking path-

ways and signals involved in routing of CD23-IgE and CD23-immune complexes to the serosal or luminal membranes are under current investigation in our laboratory.

In summary, our study has shown that human intestinal epithelial cells, both in vitro and in vivo, express CD23/Fc ϵ RII message and protein, which is regulated by IL-4. We demonstrated that the receptor is functional, being capable of binding IgE and transporting it across the epithelium. That the transport occurs in both directions fits with the concept that IgE produced within the body is transported to the apical membrane of epithelial cells and released into the lumen, at which it can interact with ingested antigens. Immune complexes can then attach to CD23, or antigen can attach to IgE-CD23, and be transported in a protected fashion inside endosomes across the epithelial barrier into the mucosa, in which IgE is released to activate effector immune (mast) cells. Our results indicate that interfering with CD23-IgE binding or reducing expression of CD23 inhibits CD23-mediated IgE transepithelial transport. This information adds to knowledge in the area of intestinal immunophysiology and may be helpful in designing novel therapies for food allergy and perhaps other allergic conditions.

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