Antigen Presentation and MHC Class II Expression by Human Esophageal Epithelial Cells

Role in Eosinophilic Esophagitis

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Professional antigen-presenting cells (APCs) play a crucial role in initiating immune responses. Under pathological conditions, epithelial cells at mucosal surfaces act as nonprofessional APCs, thereby regulating immune responses at the site of exposure. Epithelial cells in the esophagus may contribute to the pathogenesis of eosinophilic esophagitis (EoE) by presenting antigens on the major histocompatibility complex (MHC) class II. Our goal was to demonstrate the ability of esophageal epithelial cells to process and present antigens on the MHC class II system and to investigate the contribution of epithelial cell antigen presentation to EoE. Immunohistochemistry detected HLA-DR, CD80, and CD86 expression and enzyme-linked immunosorbent assay detected interferon- γ (IFN γ) in esophageal biopsies. Antigen presentation was studied using the human esophageal epithelial cell line HET-1A by reverse transcriptase-PCR, flow cytometry, and confocal microscopy. T helper cell lymphocyte proliferation was assessed by flow cytometry and IL-2 secretion. IFN γ and MHC class II were increased in mucosa of patients with EoE. IFN γ increased mRNA of HLA-DP, HLA-DQ, HLA-DR, and CIITA in HET-1A cells. HET-1A engulfed cell debris and processed ovalbumin. HET-1A cells expressed HLA-DR after IFN γ treatment. HET-1A stimulated T helper cell activation. In this study, we demonstrated the ability of esophageal epithelial cells to act as nonprofessional APCs in the presence of IFN γ . Esophageal epithelial cell antigen presentation may contribute to the pathophysiology of eosinophilic esophagitis. (*Am J Pathol* 2011, 178:744–753; DOI: 10.1016/j.ajpath.2010.10.027)

Eosinophilic esophagitis (EoE) is a unique and emerging clinicopathologic entity characterized by an intense infiltration of eosinophils into the squamous epithelium of the esophagus and is associated with basal epithelial cell hyperplasia.¹ Food hypersensitivity is implicated in the pathogenesis of EoE.² Most patients with EoE have a history of atopy.¹ The specific cytokines associated with EoE suggest a unique local T helper 2 (T_H2) phenotype.³ Increased numbers of CD4⁺ T_H lymphocytes have been observed in the esophageal mucosa of patients with EoE⁴ and also in an animal model of EoE.⁵

The epithelium of the gastrointestinal tract is the first line of defense against myriad possible antigenic substances, including food protein and commensal and pathogenic organisms. Presentation of antigen by professional antigen-presenting cells (APCs), such as dendritic cells and macrophages, is well understood. Antigen presentation by gastrointestinal tract epithelial cells may also occur under pathological conditions.^{6–8} For presentation of extracellular antigen to occur, a cell must engulf, process, and display peptides coupled to major histocompatibility complex (MHC) class II peptides on the cell membrane. In addition, the presence of costimulatory molecules on the cell surface determine whether presented antigen will provoke an immunogenic or tolerogenic T-lymphocyte response.⁹ Loss of tolerance to specific food protein may manifest as food hypersensitivity.¹⁰ Antigen presentation by epidermal keratinocytes¹¹ and both small bowel⁷ and colonic¹² epithelium is well described. Antigen presentation by intestinal epithelial cells also plays a role in food hypersensitivity,¹³ but the possibility that esophageal epithelial cells are capable of antigen presentation has not been investigated.

Proliferation of T_H lymphocytes occurs in response to antigen presentation. There are conflicting reports of changes to the number of professional APCs before and after treatment for EoE.^{4,14} Lucendo et al⁴ found that the

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number of dendritic cells was the same in normal, pretreatment EoE, and posttreatment EoE (fluticasone propionate) esophagus, whereas Teitelbaum et al¹⁴ found that the dendritic cell number was increased in the esophagus of EoE patients, compared with control.

Interferon- γ (IFN γ), although not classically associated with T_H2 diseases, is known to induce antigen presentation in multiple epithelial cell types, including epidermal keratinocytes.¹⁵ Therefore, we chose to use IFN γ to test the hypothesis that esophageal epithelial cells participate in antigen presentation by induction of the MHC class II system in our *in vitro* culture model. IFN γ mRNA may be increased in the esophageal mucosa of patients with EoE.¹⁶

In this study, we demonstrated increased IFN γ and altered epithelial cell expression of MHC class II antigens in esophageal biopsies from patients with EoE. We also demonstrated, *in vitro*, the ability of the esophageal epithelial cells to engulf, process, and present antigen to T_H lymphocytes via the MHC class II system after exposure to antigen and IFN γ . These results suggest that esophageal epithelial cells may play a central role in EoE pathogenesis by activating T_H lymphocytes through antigen presentation.

Materials and Methods

Immunohistochemistry

Grasp biopsies for routine histopathology were taken from the esophagus of pediatric patients undergoing esophagogastroduodenoscopy for upper gastrointestinal tract symptoms and were diagnosed as normal or EoE.^{1,17} Diagnosis was defined as normal for patients who had biopsies with no inflammation, no basal zone changes, and no esophageal symptoms. The EoE diagnosis was defined as ≥ 15 intraepithelial eosinophils per high power field, basal zone hyperplasia, symptoms of esophagitis, and no response to antireflux medication (as previously described).¹⁸

Biopsies were fixed in 10% neutral buffered formalin and embedded in paraffin for routine histopathology. For immunohistochemistry, embedded tissue was sectioned at 4 μ m and rehydrated; endogenous peroxidase activity was quenched with 3% H₂O₂. Nonspecific antibody binding was blocked by incubation with 5% mouse or goat serum-0.02% azide-0.02% Tween-20 in PBS. Slides were incubated overnight with goat anti-human HLA-DR (1:40; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human HLA-DP (1:40; Antigenix America, Huntington Station, NY), mouse anti-human HLA-DQ (1:40; Antigenix America), goat anti-human CD80 (15 μ g/ml; R&D Systems, Minneapolis, MN), and goat anti-human CD86 (15 μ g/ml, R&D Systems) or mouse or goat IgG as a negative control and then with biotinylated mouse anti-goat (Santa Cruz Biotechnology) or rabbit anti-mouse (Invitrogen, Carlsbad, CA) IgG secondary antibody as appropriate for 30 minutes. The species used to derive the serum was always different from the secondary antibody source species. Isotype controls were performed on separate tissue sections, to control for nonspecific secondary binding for

each patient; no positive staining was observed. Staining was performed according to the avidin-biotin-peroxidase complex method using 3,3'-diaminobenzidine as a substrate and a hematoxylin counterstain. Visualization was performed using a Nikon Eclipse TE-2000U microscope, a Nikon DXM 1200C digital camera (Nikon, Mississauga, ON, Canada) and NIS-Elements BR version 2.30 software (Nikon). Stained sections were assessed for absence or presence and localization (ie, cell type, region, and the like) of positive (ie, brown) staining.

The Health Sciences Research Ethics Board at Queen's University approved this study. All subjects and guardians provided informed written consent.

Culture Conditions

The human esophageal epithelial cell line HET-1A (CRL-2692; ATCC, Manassas, VA) is an SV-40-transformed cell line that maintains characteristics of basal esophageal epithelium. HET-1A cells were cultured in bronchial epithelial cell growth medium (BEGM; Lonza, Walkersville, MD) and treated with IFN γ , IL-4 or IL-13 (50 ng/ml; PeproTech, Rocky Hill, NJ), and dye-quenched ovalbumin (DQ ovalbumin, 15 and 30 μ g/ml; Molecular Probes, Burlington, ON, Canada) or tetanus toxoid (TT) (1 μ mol/L; List Biological Laboratories, Campbell, CA). Human embryonic kidney cell line HEK 293 was cultured in Dulbecco's modified Eagle medium (Invitrogen) with 10% fetal calf serum. Human monocytic cell line THP-1 (TIB-202; ATCC), was cultured in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% fetal calf serum. Treatment times given as 0 hours indicate immediate removal after application of treated medium.

Reverse Transcription-PCR

Total RNA was isolated from HET-1A cells using TRIzol (Invitrogen) according to the manufacturer's instructions after IFN γ , IL-4, or IL-13 incubation for 0, 24, 48, or 72 hours. Reverse transcription was performed using avian myoblast virus-reverse transcriptase (Roche, Laval, QC, Canada), followed by PCR with conditions as given in Table 1. PCR products were then visualized by electrophoresis on a 1% agarose gel.

Antigen Uptake Monitoring

HEK 293 cells were used as antigen donor cells^{19,20} that could be detected after uptake by HET-1A cells. HEK 293 cells were trypsinized and stained with PKH26 dye (μ L/ml; Sigma-Aldrich, Oakville, ON, Canada) for 2 minutes, pelleted, and resuspended in bronchial epithelial cell growth medium, snap-frozen in liquid nitrogen, thawed to 37°C, and irradiated for 20 minutes with 200,000 μ J/cm² using a CL-1000M UV Crosslinker chamber (UVP, Upland, CA), as previously described.^{19,20} After irradiation, PKH26-labeled dead HEK 293 cells in bronchial epithelial cell growth medium were added to HET-1A cultures in a 3:1 ratio for the times indicated (0, 2, 12, 24, and 48 hours). HET-1A cultures were pretreated with IFN γ (50 ng/ml) for 0, 16, 24, and 48 hours. Cells were detached

Table 1. Reverse Transcription–PCR Conditions

Gene label	Primers	T _A (°C)	Cycles (no.)
<i>HLA-DP</i>	Fwd 5′-GGAGACCGTCTGGCATCTGGA-3′ Rev 5′-CTCTCAGCGACACCTCAGT-3′	58.0	35
<i>HLA-DQ</i>	Fwd 5′-GTGCTGCAGGTGTAACTTGTACCAG-3′ Rev 5′-CACGGATCCGGTAGCAGCGGTAGAGTTG-3′	58.0	35
<i>HLA-DR</i>	Fwd 5′-AGACAAGTTCACCCACCCAG-3′ Rev 5′-AGCATCAAACCTCCAGTGCT-3′	58.0	35
<i>CIITA</i>	Fwd 5′-TTTCTGGGCACCCGCCTCAC-3′ Rev 5′-CTGGGGGAAGGTGGCTGAGA-3′	62.0	35
<i>CD80</i>	Fwd 5′-CCTCTCCATTGTGATCCTGG-3′ Rev 5′-GGCGTACACTTTCCTTCTC-3′	56.4	37
<i>CD86</i>	Fwd 5′-GTATTTTGGCAGGACCAGGA-3′ Rev 5′-GCCGCTTCTTCTTCTCCAT-3′	57.7	37
<i>GAPDH</i>	Fwd 5′-TTAGCACCCCTGGCCAAGG-3′ Rev 5′-CTTACTCCTTGAGGCCATG-3′	55.0	24

PCR cycles were 94°C for 30 seconds, the specific annealing temperature T_A for 60 seconds, and 72°C for 30 seconds. *GAPDH* was used as a loading control and water served as a negative control.

using 5 mmol/L EDTA and incubated in fluorescein isothiocyanate-conjugated anti-Ber-EP4 epithelial cell antibody (Dako Canada, Mississauga, ON, Canada) for 30 minutes at 4°C to distinguish HET-1A cells from HEK 293 cells on flow cytometry. To control for nonspecific antibody labeling, a fluorescein isothiocyanate-conjugated isotype-matched mouse plasmacytoma cell antibody was used. Cells were analyzed by flow cytometry performed using an Epics XL MCL FACS system (Beckman Coulter, Mississauga, ON, Canada) and the data were analyzed using the EXPO32 software package (Beckman Coulter). For each experiment, unlabeled and single-labeled HET-1A cells were used as controls. An immediate mixing test was used to control for background fluorescence.

For confocal microscopy, HET-1A cells were cultured on coverslips and treated with PKH26-stained dead HEK 293 cells as previously described. After 24 hours treatment, HET-1A cells were washed five times with PBS and fixed in methanol for 20 minutes at −20°C. Fixed cells were then stained with 1:50 fluorescein isothiocyanate-conjugated Ber-EP4 antibody for 60 minutes. Coverslips were mounted in PBS and staining was evaluated using an FluoView FV300 confocal microscope (Olympus, Tokyo, Japan).

Antigen Processing Assay

DQ ovalbumin (15 to 30 μg/ml; Molecular Probes) was added to HET-1A cells for 1 to 4 hours, and the cells were analyzed for increasing fluorescence, indicative of proteolytic degradation of the ovalbumin protein. THP-1, a monocytic cell line, was used as a positive control for DQ ovalbumin processing because these cells are known to degrade DQ ovalbumin under normal culture conditions.²¹ Cells were analyzed by flow cytometry as previously described.

Analysis of Membrane Protein Expression

HET-1A cells were treated with IFNγ (50 ng/ml) for 0, 16, 24, and 48 hours, harvested as previously described and

incubated for 30 minutes with phycoerythrin-conjugated antibodies to HLA-DR, CD80, and CD86 (1:100; BioLegend, San Diego, CA). Isotype-matched phycoerythrin-conjugated anti-matched mouse plasmacytoma cell antibodies (BioLegend) were used as primary antibody controls for labeling at each time point. HET-1A cells were analyzed by flow cytometry as previously described.

T-Cell Proliferation Assay

To determine the ability of antigen-loaded HET-1A cells to stimulate expansion of T_H cell populations, CD4⁺ T cells isolated from patient blood were cocultured with antigen-primed and IFNγ-stimulated HET-1A cells. Patients had been previously immunized to TT. Isolation was performed by mixing 500 μL of RosetteSep cell enrichment cocktail (Stem Cell Technologies, Vancouver, BC, Canada) with 10 ml of whole blood. After 20 minutes, the blood was diluted with an equal volume of PBS + 2% fetal calf serum and was then layered onto 20 ml of Lympholyte density-gradient cell separation medium (CedarLane, Burlington, ON, Canada) and centrifuged at 1250 × g for 20 minutes with the brake off. Enriched cells were then resuspended in PBS + 0.1% bovine serum albumin and stained for 10 minutes at 37°C with 5 μmol/L carboxyfluorescein diacetate succinimidyl ester, which becomes carboxyfluorescein succinimidyl ester (CFSE) on entry into cells. CFSE is divided equally between daughter cells during mitosis; thus, cell populations with decreased CFSE fluorescence have undergone cell division.²²

Cells were washed twice and resuspended in RPMI 1640 medium and the resulting cell suspension was added to HET-1A cultures. The isolated cells were found to be >98% CD4-positive. HET-1A cells had been primed for 48 hours with TT (1 μmol/L), and for the final 24 hours of priming, 50 ng/ml IFNγ was added to cultures to induce MHC class II expression. Before addition of T_H cells, HET-1A culture medium was removed and cells were washed three times in PBS. After 72 hours of coculture, cells in suspension were harvested, centrifuged, and resuspended for analysis. To ensure purity, cells that

did not undergo CFSE staining were resuspended in 1:200 phycoerythrin-Cy5.5-conjugated anti-CD4 antibody for 30 minutes at 4°C. Concanavalin A (5 µg/ml) was used as a positive control. Cells were analyzed by flow cytometry.

IL-2 and IFN γ Enzyme-Linked Immunosorbent Assays

Culture supernatants were collected for IL-2, and tissue was homogenized and centrifuged for 20 minutes at 2000 $\times$ g for IFN- γ enzyme-linked immunosorbent assays (ELISA, R&D Systems), which were performed in accordance with the manufacturer's instructions. Total protein was determined by the Bradford method for normalization; results are expressed as picograms per milliliter supernatant per milligram total protein.

Statistical Analysis

Numerical data are presented as means $\pm$ SEM. Differences were evaluated by one way analysis of variance (analysis of variance), followed by Newman-Keuls multiple comparison test. Significance was considered as $P < 0.05$.

Results

IFN γ , MHC Class II, and Costimulatory Molecule Expression in the Esophageal Mucosa Is Altered in EoE

ELISA demonstrated increased IFN γ expression in esophageal mucosal biopsies from patients with EoE, compared with control patient biopsies (Figure 1). IFN γ was not increased in biopsies from patients with gastroesophageal reflux disease versus control patients.

Immunohistochemistry of pediatric patient biopsies revealed differential expression of MHC class II and costimulatory proteins in esophageal inflammation. HLA-DR

Figure 1. IFN γ was significantly increased in esophageal mucosal biopsies from patients with EoE ($n = 10$) versus control patients ($n = 10$). There was no significant difference between IFN γ levels in patient biopsies between control patients and gastroesophageal reflux disease (GERD) patients ($n = 11$) and between GERD and EoE patients. Data are normalized to total tissue protein and expressed as pg IFN γ per ml per mg total protein. * $P < 0.05$; NS, not significant.

Figure 2. Negative control primary antibody slides had no visible staining (A, B). Immunostaining for HLA-DR was not found on epithelial cells from normal patients (C), but was positive on basal epithelial cells in a subset (8/19) of mucosal biopsies from patients with EoE (D, brackets and solid arrows). Intraepithelial immune cells also stained positive for HLA-DR (D, open arrows) and likely represent eosinophils ($n = 3$). Original magnification: $\times 400$ for all images.

was consistently expressed on inflammatory cells. HLA-DR was also expressed in basal epithelial cells in a subset of EoE patient biopsies (8/19 or 42%) (Figure 2D). HLA-DP and HLA-DQ were not expressed on esophageal epithelial cells under either normal or EoE conditions (data not shown), although light staining was observed on intraepithelial inflammatory cells. Most of these cells had visible bilobed nuclei characteristic of eosinophils, which are known to express HLA-DR.²³

Expression of costimulatory molecule CD80 was localized to the plasma membrane of esophageal epithelial cells in normal biopsies, but was not observed on epithelial cells in EoE biopsies (Figure 3A). CD86 was expressed on the epithelial cell surface of biopsies from EoE patients, but not in normal esophageal biopsies (Figure 3B).

IFN γ Increases Antigen Presentation-Associated mRNA Expression in HET-1A Cells

To determine whether antigen presentation in HET-1A cells could be altered by IFN γ , mRNA was isolated from IFN γ -treated and untreated cells (Figure 4). Increased mRNA expression for HLA-DR, HLA-DP, HLA-DQ, and CIITA were observed after IFN γ treatment. The increase in HLA-DP and HLA-DR mRNA was sustained for 72 hours after IFN γ treatment, whereas HLA-DQ and CIITA mRNA had returned to basal levels by 72 hours after treatment. Transcripts for the costimulatory molecule CD80 were constitutively expressed and were not altered by IFN γ treatment. CD86 transcripts decreased as IFN γ

Figure 3. Costimulatory molecules CD80 and CD86 were differentially expressed on esophageal mucosal biopsies. No staining was present in isotype control goat IgG (glgG) for CD80 primary antibody (**A** and **B**). Human CD80 (hCD80) costimulatory molecule expression was found on biopsies from normal patients (**C**), but this expression was not present on EoE patient biopsies (**D**, $n=3$). No staining was present in isotype control goat IgG for CD86 primary antibody (**E** and **F**). Human CD86 (hCD86) was not expressed on normal patient biopsies (**G**), but was found on epithelial cells in biopsies from patients with EoE (**H**, $n=3$). Original magnification: $\times 400$ for all images.

incubation time increased. All transcripts were found at the expected length for the primers used. No transcripts were detected in lanes where cDNA was replaced with H_2O (negative control). Expression of the housekeeping gene GAPDH was consistent for each experimental condition.

HET-1A Cells Engulf and Process Antigen

To study the ability of HET-1A cells to engulf antigen, HEK 293 cells were stained with PKH26 dye, lysed, UV-irradiated, and introduced into HET-1A culture medium. PKH26 is a lipophilic dye able to specifically label cells; it maintains its fluorescent properties without transfer to other cells in culture.²⁴ Flow cytometric detection of dead HEK 293 cell uptake is an established model for demonstration of antigen uptake.^{19,20} To set up proper gates for

this experiment, an immediate control and a 24-hour time point were performed (**Figure 5A**). To identify HET-1A cells, events were then gated by positive labeling with fluorescein isothiocyanate-conjugated anti-Ber-EP4 epithelial cell antibody (**Figure 5A**, third panel), and a gate was established for HET-1A cells that were PKH26-positive, based on the differences between the immediate control and the 24-hour time point (**Figure 5A**, fourth panel). Dead HEK 293 cells engulfed by HET-1A cells after 24 hours were visualized using confocal microscopy (**Figure 5B**).

Flow cytometric analysis demonstrated the time-dependent ability of HET-1A cells to engulf dead HEK 293 cells (**Figure 5C**) after 12 hours of treatment ($32.8 \pm 7.3\%$ PKH26-positive HET-1A cells), which increased to maximal uptake ($56.0 \pm 1.5\%$) after 48 hours of treatment. Pretreatment with $IFN\gamma$ (50 ng/ml) for ≥ 16 hours diminished, but did not abolish, the percentage of HET-1A cells engulfing dead HEK 293 cells over 24 hours (**Figure 5D**).

After uptake of exogenous proteins, APCs must process extracellular proteins into peptide fragments²⁵ before loading onto MHC class II proteins for presentation. The ability of HET-1A cells to process proteins into peptide fragments was analyzed by breakdown of ovalbumin conjugated to a self-quenching dye (DQ ovalbumin; Molecular Probes), which was added to culture medium for 1 to 4 hours before analysis by flow cytometry. HET-1A esophageal epithelial cells degraded DQ ovalbumin in a time-dependent manner between 0 and 4 hours (**Figure 6A**). Fluorescent mean after 4 hours in $30 \mu\text{g/ml}$ was 56.3 ± 10.2 ($P < 0.05$ compared with immediate control, for all conditions). Pretreatment of HET-1A with $IFN\gamma$ (50 ng/ml) did not alter the ability of HET-1A cells to process DQ ovalbumin (**Figure 6B**). The immediate control (0 hours) had a fluorescent mean at 29.3 ± 7.2 ; at 16 hours, the fluorescent mean was 38.7 ± 4.9 ($P < 0.05$).

Figure 4. RT-PCR demonstrated increased mRNA expression of HLA-DR, HLA-DQ, HLA-DP, and CIITA after $IFN\gamma$ treatment (50 ng/ml) of HET-1A cells for 24, 48, and 72 hours. Costimulatory molecule CD80 and CD86 expression was not increased by $IFN\gamma$. GAPDH was used as a loading control and H_2O as a negative control. Representative images are shown ($n = 3$). PCR product lengths are indicated at the left.

Figure 5. HET-1A cells engulf dead HEK 293 cells. **A:** Flow cytometry data acquisition, **left to right:** At 10 minutes after application of PKH26-stained, dead HEK 293 cells to HET-1A cultures, separate populations were present and no cells were double positive (**first panel**). At 24 hours, populations of double-positive PKH26⁺/Ber-EP4⁺ cells were present; these are HET-1A cells that have engulfed dead HEK 293 cells (**second panel**). Next, only cells staining for esophageal epithelial cell marker Ber-EP4 were gated (**third panel**). The final gate shows the HET-1A cells that had engulfed killed HEK 293 cells (results expressed subsequently as a percentage), and was drawn based on the immediate control versus 24 hours treatment (**fourth panel**). Representative plots are shown ($n = 3$). **B:** Composite confocal micrograph demonstrating HET-1A cell (green) uptake of dead HEK 293 cells (red) over 24 hours. **Arrows** indicate where the HEK 293 cells had been engulfed by HET-1A cells (yellow). The image is a representative composite of five serial z-slices, 3 μm thickness each, showing intracellular staining of dead HEK 293 cells. Original magnification: $\times 400$. **C:** Time course of treatment of HET-1A cells with dead HEK 293 cells ($n = 3$). $*P < 0.05$. **D:** Pretreatment with IFN γ (50 ng/ml) decreased, but did not abolish, the uptake of dead HEK 293 cells by HET-1A cells over 24 hours ($n = 3$). $*P < 0.05$ for the 0-hour time point compared with subsequent time points.

IFN γ Induces HET-1A Cell MHC Class II Expression

HET-1A cells were labeled with fluorescent antibodies directed against the MHC class II gene product HLA-DR and costimulatory molecules CD80 (B7-1) and CD86 (B7-2). Flow cytometry revealed that HET-1A cells do not constitutively express HLA-DR, but increasingly express this protein on the cell surface after stimulation with IFN γ (**Figure 7A**). Although mRNA for costimulatory molecules CD80 and CD86 was present (**Figure 3**), surface expression of CD80 and CD86 protein was not detected by flow cytometry (**Figure 7, B and C**). Similar experiments using flow cytometry with IL-4 treatment (50 $\mu\text{g}/\text{ml}$) in place of IFN γ did not affect HLA-DR expression (data not shown).

Treatment of HET-1A Cells with IFN γ and Priming with Tetanus Toxoid Induces Proliferation of T_H Lymphocytes from Tetanus-Immunized Subjects

To demonstrate that HET-1A cells are able to elicit an antigen-specific T_H -lymphocyte response, we cocultured T_H cells isolated from peripheral blood of subjects previously immunized against tetanus with HET-1A cells pre-treated with tetanus toxoid and IFN γ . The T_H cells cocultured with HET-1A cells that had been primed with tetanus toxoid and treated with IFN γ proliferated more ($21.0 \pm 1.9\%$ divided cells) than did those with neither treatment ($10.0 \pm 1.2\%$) or with IFN γ ($11.5 \pm 1.0\%$) or TT

Figure 6. HET-1A cells process DQ-ovalbumin. **A:** HET-1A cells were able to process DQ ovalbumin into fluorescent peptides in a concentration- and time-dependent manner, as indicated by flow cytometry ($n = 3$). All treatments $P < 0.05$, compared with untreated. THP-1 monocytic cells were used as a positive control to verify DQ ovalbumin processing. **B:** HET-1A cells, analyzed by flow cytometry, processed DQ ovalbumin into peptides independent of IFN γ treatment (50 ng/ml, $n = 3$). All treatment conditions were significantly increased over untreated control, but did not differ significantly from one another (NS, not significant).

Figure 7. IFN γ induced HLA-DR expression on HET-1A cells. **A:** HET-1A cells expressed HLA-DR only after IFN γ treatment (50 ng/ml) for 16 to 48 hours as shown by flow cytometry ($n = 3$). Over 16 to 48 hours, IFN γ (50 ng/ml) treatment did not induce detectable CD80 (**B**) or CD86 (**C**) expression on HET-1A cell surface ($n = 3$).

($12.6 \pm 1.8\%$) alone (Figure 8A). The T_H-cell proliferation was measured in terms of decreasing CFSE fluorescence. Concanavalin A ($35.6 \pm 5.1\%$) was used as a positive control, because it is known to elicit T_H-cell activation. Presumably, IFN γ was required to induce antigen-loaded MHC class II expression on the cell surface (Figure 6A).

Production of IL-2 in HET-1A and T_H-cell coculture supernatants provided additional evidence of T_H-cell activation. IL-2 secretion was induced with combined IFN γ and TT treatment (6.6 ± 1.4 pg/ml per mg protein) and with concanavalin A treatment (24.9 ± 6.7 pg/ml per mg protein, positive control), compared with no treatment (0.6 ± 0.1 pg/ml per mg protein) or with either IFN γ (0.7 ± 0.2 pg/ml per mg protein) or TT (0.8 ± 0.2 pg/ml per mg protein) treatment alone (Figure 8B).

Interleukin-4 Increases HET-1A Costimulatory Molecule CD80 Expression

HET-1A cells treated for 0, 24, 48, and 72 hours with IL-4 (Figure 9A) or with IL-13 (Figure 9B) had unchanged expression of antigen presentation-associated genes for HLA-DR and CD86, but CD80 expression was altered. Costimulatory molecule CD80 mRNA was increased after IL-4 treatment and decreased after IL-13 treatment. IL-4 induced a maximal response at 48 hours, but its effect had decreased by 72 hours. IL-13 initially decreased CD80 expression at 24 and 48 hours, but its effect was also diminished at the 72-hour time point. Consistent expression of the housekeeping gene GAPDH was used as an internal control.

Discussion

In this study, we have demonstrated that esophageal epithelial cells are capable of acting as nonprofessional APCs in the context of increased IFN γ and induction MHC class II expression. Antigen presentation to T_H lymphocytes is the initiating event in the highly specific immune response to foreign antigens. Antigen presentation by epithelial cells in the gastrointestinal tract plays an important role in pathological processes such as Crohn's disease.²⁶ Similarly, antigen presentation by esophageal epithelial cells may contribute to the pathophysiology of eosinophilic esophagitis.⁴ The presence of MHC class II protein HLA-DR on esophageal epithelium has been previously described in patients with Crohn's esophagitis²⁷ and herpes simplex virus-induced esophagitis.²⁸ The contribution of epithelial expression of MHC class II to esophageal disease is not known, but these studies raise the possibility that epithelial cells may act as nonprofessional APCs. In the present study, immunohistochemical staining for HLA-DR was positive on epithelial cells in a subset of biopsies from patients with EoE, but not in normal patient biopsies. We found no demographic differences (age, sex, symptoms, endoscopic features, atopic status, and histological features) in the subset of EoE patients expressing HLA-DR, compared with those who did not. It is possible that patients with EoE differ in MHC class II expression because of the duration of disease or other factors.

Eosinophilic esophagitis is associated with food allergy,¹ although the mechanism by which foods contribute to EoE is unknown. EoE is characterized by increased

Figure 8. IFN γ -stimulated, antigen-primed HET-1A cells induced T_H-lymphocyte activation. **A:** T_H cells isolated from tetanus toxoid (TT)-immunized patient blood proliferated at a basal level in coculture with HET-1A cells, as measured by decreasing CFSE fluorescence, and proliferation was increased by combined IFN γ (50 ng/ml) and antigen (TT, 1 μ mol/L) treatment, but not by either treatment individually. Concanavalin A (Con A) was used as a positive control ($n = 3$). * $P < 0.05$. **B:** IL-2 concentration, normalized to protein concentration, in coculture supernatants (from experiments presented in panel A) was significantly increased by combined IFN γ and antigen (TT) treatment, as determined by ELISA ($n = 3$). * $P < 0.05$.

Figure 9. A: RT-PCR demonstrating that treatment of HET-1A cells with IL-4 (50 ng/ml) for 0, 24, 48, and 72 hours did not alter expression of HLA-DR or CD86. IL-4 did increase mRNA expression of CD80 after 24 hours. B: Treatment of HET-1A cells with IL-13 (50 ng/ml) for 0, 24, 48, and 72 hours also did not alter expression of HLA-DR or CD86. CD80 expression was markedly decreased after 24 hours of IL-13 treatment. GAPDH was used as a loading control and H₂O as a negative control ($n = 3$). Representative images are shown. All bands were located at the expected base-pair lengths.

eosinophils, basal hyperplasia of the nonkeratinized stratified squamous epithelium,^{18,29} increased intraepithelial CD4⁺ T_H lymphocytes,⁴ and possibly increased local production of IFN γ .¹⁵ Dendritic cells are also present in small numbers in the esophagus.³⁰ These professional APCs do not differ significantly between normal and EoE patients.⁴ It is unknown whether dendritic cells or other, nonprofessional APCs, such as esophageal epithelial cells, are responsible for the increase in T_H cells seen in EoE. In the small intestine, competition for antigen presentation between dendritic cells and epithelial cells is known to occur.³¹ Although dendritic cell number is unchanged in EoE, basal epithelial cell number is significantly increased,¹⁸ and the basal epithelial cells appear also to express MHC class II in patient biopsies. With this background in mind, we investigated the ability of esophageal epithelial cells to act as nonprofessional APCs leading to a specific T_H-lymphocyte immune response.

In the present study, we found that IFN γ is increased in mucosal biopsies from patients with EoE and that antigen presentation by esophageal epithelial cells is dependent on IFN γ . In the small bowel and colon, epithelial MHC class II expression and antigen presentation is induced by IFN γ and contributes to the loss of tolerance to luminal antigen observed during inflammatory bowel disease and food allergy.^{32,33} IFN γ is also known to regulate antigen presentation in multiple cell types, including epidermal keratinocytes.³⁴ Gupta et al¹⁶ found elevated levels of IFN γ mRNA in the esophageal mucosal biopsies of patients with EoE, by real-time PCR, although no internal control gene was used. From gene array data of patients with EoE, Blanchard et al³⁵ identified upregulation of five genes that are specifically induced by IFN γ . Taken to-

gether, these findings suggest that IFN γ may play a role in the pathogenesis of EoE.

Even though IFN γ (classically a T_H1-associated cytokine) is increased in EoE, many of the cytokines increased during EoE are members of the T_H2 family.³⁵ Overall, the cytokine profile of EoE does not conform to the classic T_H1 versus T_H2 model of cytokine expression and so has been termed adaptive.³⁶ We postulate that IFN γ may be one of multiple factors that interact to initiate antigen presentation by esophageal epithelial cells in EoE. In contrast to a previous report, we did not find a significant increase in IFN γ concentration in gastroesophageal reflux disease biopsies, compared with control patient biopsies.³⁷

In the present study, IFN γ increased the expression of mRNA for HLA-DR, HLA-DP, HLA-DQ, and CIITA in the esophageal epithelial cell line HET-1A. The finding that HET-1A cells do not express HLA-DR under normal culture conditions, but up-regulate its expression in response to IFN γ , implies that MHC class II antigen presentation by this cell type occurs during immune activation. We observed the MHC class II protein HLA-DR on basal epithelial cells in EoE biopsies, but not in normal patient biopsies.

HET-1A cells were shown to engulf and process antigen. Antigen uptake, as measured by HET-1A uptake of dead HEK cells, was decreased by IFN γ treatment. Similar down-regulation of antigen uptake by IFN γ has been demonstrated in macrophages.^{21,38} Dye-quenched ovalbumin processing occurred rapidly in HET-1A cells and appeared to be independent of IFN γ .

HET-1A cells primed with tetanus toxoid antigen were able to induce T_H-cell proliferation and increase IL-2 secretion only after treatment with IFN γ and tetanus toxoid. These experiments indicate a possible link between the inflammatory cytokines present in esophageal inflammation and the increased number of intraepithelial CD4⁺ cells (T_H lymphocytes)⁴ seen in EoE patient biopsies. The etiology of EoE is poorly understood, although indirect evidence from studies investigating elimination of specific foods and elemental diet³⁹ suggests that the pathogenesis of EoE is related to exposure to specific food protein. Thus, the pathogenesis of EoE may be mediated by presentation of food protein by epithelial cells to T_H lymphocytes. It could be worthwhile in future studies to investigate this phenomenon using T_H cells isolated from EoE patients.

Costimulatory molecules increase the physical and temporal interaction between the T-cell receptor and the antigen-MHC class II complex.⁴⁰ In the present study, CD86 immunostaining was seen in EoE biopsies, but not in normal control biopsies. HET-1A cells were able to elicit a T_H-cell response to antigen despite the absence of classic costimulatory molecules as determined by flow cytometry of IFN γ -stimulated cells. Induction of CD80 or CD86 expression on HET-1A cells may occur as the result of other proinflammatory cytokines (another promising subject for future studies). Other costimulatory molecules, such as the inducible T-cell costimulator, have been discovered and found to aid in T_H-cell activation in a similar manner to CD80/86,⁴¹ although their function is

not well characterized. Evidence exists from other studies demonstrating that epidermal keratinocytes in culture provide an effective signal for costimulation, resulting in T-cell proliferation, which is outside the traditional CD80/86 pathway.⁴² It is also possible that the process of isolating and culturing T_H cells increases their responsiveness to antigen.

In conclusion, we have found that esophageal epithelial cells express MHC class II *in vivo* and that this expression is altered in EoE. We have demonstrated that esophageal epithelial cells are able to engulf, process, and present antigen and to stimulate T_H-lymphocyte activation by presented antigen. Although a multitude of cytokines may be involved in EoE, these mechanisms are regulated in part by the inflammatory cytokine IFN γ , which is increased in the esophageal mucosa of patients with EoE. These findings support a role for nonprofessional antigen presentation by esophageal epithelial cells in the pathogenesis of EoE.

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