# Isolation of Human Conjunctival Mast Cells and Epithelial Cells: Tumor Necrosis Factor-α from Mast Cells Affects Intercellular Adhesion Molecule 1 Expression on Epithelial Cells

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PURPOSE. To isolate and purify mast cells and epithelial cells from human cadaveric donor conjunctival tissue and to characterize interactions between these cell types in vitro.

METHODS. Monodispersed cell suspensions obtained by enzymatic digestion of conjunctival tissue were applied to a single-density Percoll gradient. Epithelial cells obtained from the top layer of the gradient were cultured to confluence. Mast cells obtained from the pellet were equilibrated in culture medium and further purified using a two-step Percoll gradient. Using reverse transcription-polymerase chain reaction (RT–PCR), RNA from the purified mast cell preparation was probed for tumor necrosis factor-α (TNFα) message. Fluorescence activated cell sorting (FACS) analysis of intracellular immunostained mast cells was used to detect the TNFα protein. An examination for intercellular adhesion molecule 1 (ICAM-1) on epithelial cells was performed after 24-hour incubations with either recombinant TNFα supernatants from calcium ionophore A23187 (CaI)-stimulated mast cells or appropriate controls using FACS analysis.

RESULTS. Highly purified human conjunctival mast cells and epithelial cells (each >95%) were obtained from human cadaveric donor tissue. RT–PCR analysis of purified mast cell RNA revealed the expression of TNFα mRNA. An evaluation of mast cells for intracellular protein demonstrated positive staining for tryptase and TNFα. ICAM-1 was found on purified epithelial cells, and incubation of epithelial cell monolayers with supernatants from CaI-stimulated mast cells resulted in upregulation of this receptor. This upregulation was blocked by incubation with TNFα-neutralizing antibody.

CONCLUSIONS. This work provides the methods for isolating and purifying mast cells and epithelial cells from human donor tissue and the opportunity for studying mechanisms of conjunctival inflammation by evaluating the interactions between these cells. (*Invest Ophthalmol Vis Sci.* 1998; 39:336–343)

Conjunctival mast cells and their mediators are implicated, at least in part, in all types of allergic ocular disease. Human studies of allergic ocular disease, however, have primarily consisted of the study of pathologic biopsy specimens (demonstrating increased numbers of mast cells, eosinophils, and other inflammatory cells) and tear film evaluation for mediators and cellular infiltration.[1-3] To date, a mechanistic approach to the biologic interactions between mast cells and human ocular surface cells has not been undertaken. Specifically, the effect of human conjunctival mast cells and their mediators on either the conjunctival epithelium and fibroblasts or the corneal epithelium and fibroblasts has not been investigated.

The recent finding of cytokines in tissue- and bone-marrow-derived mast cells has generated a heightened interest in the role of the mast cell in allergic conditions.[4,5] In addition, the epithelium of tissues undergoing allergic reactions is viewed more as an active, rather than passive, participant in these reactions. In this article, we extend our previous work[6] demonstrating mast cell isolation from human conjunctival tissue by describing the purification of human conjunctival mast cells and epithelial cells from cadaveric donor tissue pools. We further report that tumor necrosis factor-α (TNFα), found in purified conjunctival mast cells, affects epithelial cell intercellular adhesion molecule-1 (ICAM-1) expression. This work provides a model to examine mechanistically the effect of mast cell-derived mediators on ocular surface epithelial cells and fibroblasts.

## MATERIALS AND METHODS

### Reagents and Solutions

Collagenase (type I), hyaluronidase (type I-S), trypsin–EDTA, Hepes, Triton X-100, Alcian blue, trypan blue, Percoll, fetal calf

serum, RPMI 1640, gentamicin, penicillin-streptomycin, amphotericin, Hanks' balanced salt solution (without $Ca^{2+}$, $Mg^{2+}$, or phenol red) (HBSS), bovine serum albumin, $NaN_3$, phenylmethylsulfonyl fluoride, mouse anti-pan cytokeratin antibody, goat anti-human immunoglobulin E (IgE) antibody, and actinomycin D were obtained from Sigma Chemical (St. Louis, MO). Keratinocyte growth medium was obtained from Clonetics (San Diego, CA). Wright's stains were performed using a staining kit (Diff-Quik; Baxter Scientific Products, McGaw Park, IL). Calcium ionophore A23187 (CaI) was obtained from Calbiochem, Novabiochemical (La Jolla, CA); a histamine radioimmunoassay kit was obtained from Immunotech (Westbrook, ME); rabbit anti-chicken immunoglobulin Y-fluorescein isothiocyanate (IgY-FITC) polyclonal antibody was obtained from Jackson ImmunoResearch (West Grove, PA); mouse anti-human TNFα-phycoerythrin (PE) monoclonal antibody was obtained from PharMingen (San Diego, CA); mouse anti-human ICAM-1-PE monoclonal antibody was obtained from Becton-Dickinson (San Jose, CA); goat anti-human TNFα neutralizing polyclonal antibody was obtained from R&D Systems (Minneapolis, MN); and recombinant TNFα was obtained from Genzyme Diagnostics (Cambridge, MA). Isotype controls for FACS antibodies were purchased from the same manufacturer as the respective antibodies. The chicken anti-human tryptase IgY polyclonal antibody was a generous gift from Promega (Madison, WI). Total RNA was isolated using a reagent (Ultraspec RNA; BIOTECX Laboratories, Houston, TX).

For reverse transcription-polymerase chain reaction (RT-PCR), the following reagents were used: recombinant RNAsin (Promega), reverse transcriptase (Superscript II; Life Technologies, Gaithersburg, MD), *Taq* DNA polymerase (Promega), a primer pair for TNFα (Clontech), and a blotting kit (ECL System Southern; Amersham, Arlington Heights, IL).

The Tyrode's physiological salt solution plus gelatin (TG) used in these studies consisted of 137 mM NaCl, 2.6 mM KCl, 0.35 mM $NaH_2PO_4$, 11.9 mM $NaHCO_3$, 5.5 mM glucose, 1 g/l gelatin (adjusted to pH 7.4 with HCl). TGCM is TG with added $CaCl_2$ (2 mM) and $MgCl_2$ (1 mM). Percoll stock solution was prepared by mixing the commercial Percoll solution and 10× Hepes buffer plus $dH_2O$ to obtain an osmolality of 285 mOsm/kg $H_2O$. The desired densities of Percoll were prepared by mixing the Percoll stock solution with TG as described by Undem and coworkers.[7] HBSS-BAP, used as a FACS-staining buffer, consisted of HBSS, bovine serum albumin (1 g/l), $NaN_3$ (0.5 g/l), and phenylmethylsulfonyl fluoride (18 mg/l in 2 ml ethanol).

## Mast Cell and Epithelial Cell Isolation, Purification, and Culture

Human conjunctival tissue was obtained with permission from organ and tissue donors. Upper and lower bulbar conjunctivas were aseptically collected within 8 hours of death (average time, 4.5 hours) and transported in corneal preservation medium (Dexsol; Chiron Ophthalmics, Irvine, CA). Tissue acquisition protocols were approved by the University of Wisconsin Human Subjects Committee. Cadaveric conjunctival tissues were resuspended on arrival in mast cell culture medium (RPMI 1640 supplemented with 20% fetal calf serum, 2.5 mM L-glutamine, 400 μg/ml streptomycin, 20 μg/ml gentamicin, and 5 μg/ml amphotericin) for as many as 4 or 5 days and stored at 4°C until four to eight sets had been received. This number of tissues was found routinely to yield enough mast cells and epithelial cells to conduct one experiment. In the

isolation procedure, sets of tissues were transferred to petri dishes, equilibrated at 37°C overnight, blotted, and weighed to determine the amount of enzyme required for digestion. Hyaluronidase and collagenase were used to digest the tissue. The digestion process (37°C for 30 minutes on a rotating shaker) was first performed at a low concentration of enzymes (two digestions at 200 U/g in a 10-ml final volume). This was followed by digestion at a high concentration of enzymes (three to six digestions at 2000 U/g in a 10-ml final volume). Each digestion was followed by washing of tissue in TGCM over a 100-μm, nylon-mesh filter to collect freed cells. The freed cells were then centrifuged, resuspended in TG, pooled, layered over two single-density 10-ml Percoll gradients (density, 1.041 g/ml), and centrifuged for 20 minutes at 800g. After centrifugation, the top layers of the gradients (epithelial cells) were harvested, washed, and resuspended in keratinocyte growth medium (without hydrocortisone; concentration, $1 \times 10^6$ cells/ml) and were transferred to a collagen-fibronectin-coated, 24-well plate (0.5 ml/well) for culturing at 37°C. The medium was changed every 48 hours until confluence. Purity was determined by cytokeratin staining using FACS analysis. Pelleted cells from the gradients (mast cells) were washed and resuspended to a concentration of $1 \times 10^6$ cells/ml in mast cell culture medium, transferred to a 24-well plate (0.5 ml/well), and equilibrated at 37°C. After equilibration, the cells were removed from the plate with gentle flushing and scraping and were suspended in TG. The cells were then washed, resuspended in 1 ml TG, and layered over a two-step Percoll gradient (densities of 1.08 g/ml on top of 1.123 g/ml). This gradient was centrifuged for 20 minutes at 800g. Mast cells were harvested from the interface of the two Percoll densities and were washed and resuspended in TGCM for challenge. Morphology of the purified mast cells was evaluated by electron microscopy carried out by the Electron Microscopy Unit at Alcon Laboratories. Differentiation of other cells was determined on cytospins using Wright's stain.

## Challenge of Mast Cells

All mast cell challenges were conducted in TGCM buffer at minimum concentrations of $5 \times 10^3$ mast cells/tube (in duplicate). Reagents used for challenge (CaI [10 μg/ml], goat anti-human IgE antibody [1 and 10 μg/ml], and goat IgG antibody control [10 μg/ml]) were diluted in TGCM to appropriate concentrations and added to cells at volumes of 10 μl/ml cells. The doses chosen were based on the previous characterization of semipurified conjunctival mast cell preparations.[6] Cell incubations were performed in a 37°C shaking water bath for 30 minutes. After incubation, the tubes were removed, placed on ice, and pelleted in a centrifuge (Sero-Fuge II; Clay Adams, Parsippany, NJ). Total histamine content of cell suspensions was determined after treatment with an equal volume of 0.4% Triton X-100 detergent to lyse the cells. Supernatants were removed and frozen at −70°C until analysis. Histamine analysis was performed by radioimmunoassay at dilutions of 1:5.

## Purified Conjunctival Mast Cells: RNA Extraction and Tumor Necrosis Factor-α Reverse Transcription–Polymerase Chain Reaction

Purified mast cell pellets were homogenized and frozen at −70°C until use. RNA was extracted from thawed and pooled homogenates (a total of $1.6 \times 10^5$ mast cells at >95% purity) using the Ultraspec RNA reagent according to the manufactur-

er's instructions. RNA was reverse transcribed as previously described.[8] Briefly, 8 $\mu$l of the isolated RNA was incubated with 0.5 $\mu$g of random primers (70°C for 2 minutes). This RNA-primer mixture was then incubated with 200 U reverse transcriptase, 4 $\mu$l 5× reaction buffer, 0.01 M dithiothreitol, 40 U recombinant RNAsin, and 0.5 mM of each dNTP in a total volume of 20 $\mu$l (37°C for 1 hour). This cDNA-containing solution was diluted 1:3 in water, and 4 $\mu$l was transferred to a 650-$\mu$l, thin-walled PCR tube containing 2 U *Taq* DNA polymerase, 5 $\mu$l 10× PCR buffer, 3 mM MgCl$_2$, 0.2 mM of each dNTP, and 0.4 $\mu$M of each primer (see below) in a total reaction volume of 50 $\mu$l. The primer pair for TNF$\alpha$ (upstream primer, GAGTGACAAGCCTGTAGCCCATGTTGTAGCA; downstream primer, GCAATGATCCCAAAGTAGACCTGCCCAGACT), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (upstream primer, TGAAGGTCGGAGTCAACGGATTTGGT; downstream primer, CATGTGGGCCATGAGGTCCACCAC) spanned introns, and no amplification of genomic sequences was observed. The PCR mixture was overlaid with oil, denatured by heating to 94°C for 5 minutes, and subjected to 26 cycles (TNF$\alpha$) or 24 cycles (G3PDH) of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and then extension at 72°C for 2 minutes. Controls in each PCR run included a sample containing reagents but no cDNA (negative control) and samples containing TNF$\alpha$ or G3PDH cDNA templates (positive controls). The PCR products were electrophoresed onto a 2.0% agarose gel, and the identity of the PCR products was verified by Southern blotting using a commercial kit (ECL; Amersham) according to the manufacturer's instructions.

## Fixation and Permeabilization of Conjunctival Cells

The method used was a modification of the methods published by Schmid and coworkers.[9] In this procedure, nonionic detergent is used to increase the permeability of the cell membrane. Fixation was accomplished by resuspending cells in 0.5 ml cold phosphate-buffered saline and 0.5 ml cold 2% paraformaldehyde solution. The samples were then vortexed, incubated at 4°C for 1 hour, and centrifuged for 3 minutes, and the supernatant was decanted. For permeabilization, the fixed cell pellet was resuspended in 1 ml 0.2% Tween 20 in phosphate-buffered saline and incubated for 15 minutes at 37°C. One milliliter phosphate-buffered saline supplemented with 2% newborn calf serum and 0.1% NaN$_3$ was then added to the cells. This suspension was centrifuged (3 minutes), and the pellet was resuspended in HBSS-BAP and divided for staining.

## FACS Analysis of Mast Cells for Tryptase and Tumor Necrosis Factor-$\alpha$

To guarantee that a large number of mast cells was analyzed for TNF$\alpha$, semipurified mast cell preparations (50% mast cells) were used for FACS analysis. To assure that cells, analyzed for TNF$\alpha$, were indeed mast cells, a two-color staining technique was used. In this procedure, cells to be studied were stained in the same tube for mast cell-specific tryptase (fluorescein isothiocyanate [FITC]) and TNF$\alpha$ (phycoerythrin). The tryptase antibody used was a polyclonal chicken IgY. A second FITC-labeled, rabbit anti-chicken IgY antibody was used to detect the anti-tryptase-labeled cells. Phycoerythrin was directly conjugated to the anti-TNF$\alpha$ antibody. Species- and chromagen-appropriate isotype antibody controls were included for each

antibody used. In this specific procedure, the conjunctival cell preparation was washed and resuspended in HBSS-BAP to a concentration of 1 × 10$^6$ cells/ml, and the anti-tryptase antibody was added at a volume of 10 $\mu$l/tube (1:50 dilution). Ten microliters per tube each of rabbit IgG and mouse IgG (1:10 dilution) was added to protect against the nonspecific sticking of antibody, and the mixture was then incubated on ice for 30 minutes on a rotating shaker. After incubation, the cells were washed twice in 2 ml HBSS-BAP; resuspended in a 1:50 dilution of the FITC-labeled, rabbit anti-chicken IgY (100 $\mu$l/tube) and 10 $\mu$l/tube PE-labeled anti-TNF$\alpha$ antibody and incubated as described above. After the second incubation, the cells were washed and resuspended in HBSS-BAP (500 $\mu$l/tube) for FACS analysis. The cells were analyzed using a histogram region gated on the tryptase-positive cells. Results are expressed as the percentage of tryptase-positive (mast) cells that were also cytokine positive.

## FACS Analysis of Conjunctival Epithelial Cells for Cytokeratin and Intercellular Adhesion Molecule 1

Confluent monolayers of epithelial cells (one passage) were harvested with trypsin-EDTA and resuspended in HBSS-BAP to a concentration of 5 × 10$^5$ cells/ml. Cells to be stained for intracellular cytokeratin were fixed, permeabilized, and stained with an FITC-conjugated, mouse anti-pan cytokeratin antibody or an FITC-conjugated, mouse IgG1 isotype control (20 $\mu$l/tube). For ICAM-1 analysis, cells (viable, not fixed) were stained with PE-labeled mouse anti-ICAM-1 antibody and a PE-labeled mouse IgG2a isotype control at amounts recommended by the manufacturer (20 $\mu$l/tube). The cells were added in a volume of 100 $\mu$l/tube (50,000 cells) and incubated on ice for 30 minutes. After washing, they were resuspended in 500 $\mu$l/tube of HBSS-BAP, and propidium iodide was added to each tube to determine viability. The resultant histograms were analyzed geometrically and based on gating of live cells only. Values in median fluorescence units (MFU) were generated for statistical comparison by subtracting median fluorescence of isotype control-stained cells from median fluorescence of anti-ICAM-1-stained cells.

When cytokine upregulation of ICAM-1 was examined, the cytokine was added at a concentration of 200 U/ml (10 $\mu$l/well in a 24-well plate with 0.5 ml medium/well) and incubated for 24 hours at 37°C before harvesting. Each treatment included eight wells per cytokine. The cells were divided after harvesting to facilitate staining with either anti-ICAM-1 or the isotype antibody control. To obtain mast cell supernatants, purified mast cells (>95%) were incubated with CaI (10 $\mu$g/ml) for 30 minutes at 37°C. When CaI-conditioned mast cell supernatants were used, the supernatant or controls were combined 1:1 with keratinocyte growth medium and added to the wells. To determine the contribution of TNF$\alpha$ in mast cell supernatants to upregulation of epithelial cell ICAM-1, goat anti-human TNF$\alpha$-neutralizing antibody was used. Doses of neutralizing antibody used were predicted using the ND$_{50}$ level determined by the manufacturer. The CaI-conditioned mast cell supernatants were incubated with two doses of neutralizing antibody (0.02 $\mu$g/ml and 0.2 $\mu$g/ml) for 1 hour at 37°C. Controls consisted of epithelial cells with the following treatments: unstimulated (constitutive ICAM-1 expression), TNF$\alpha$ (5 ng/ml), TNF$\alpha$ (5 ng/ml) plus neutralizing antibody (50 $\mu$g/ml), CaI (10 $\mu$g/ml), and CaI-conditioned mast cell supernatants plus goat IgG (50 $\mu$g/ml). To determine the baseline expression of

ICAM-1, actinomycin D (2 μg/ml) treatment of monolayer epithelial cells was performed. A comparison of the unstimulated epithelial cells with the baseline epithelial cell ICAM-1 expression (actinomycin D-treated) indicated the level of ICAM-1 upregulation in epithelial cells caused by the endogenous release of mediators during the 24-hour incubation period. The effectiveness of the drug dose was evaluated by adding TNFα with the drug to the epithelial cell monolayer.

## Data Analysis

All statistical analyses were performed using Systat Version 6 software (Systat, Evanston, IL). Treatments were compared using a general, linear-model, analysis-of-variance-randomized block model with experiments as blocks. Planned post hoc comparisons were made with Fisher least-significant-difference tests. Residuals were checked for abnormal distribution with a probability plot and Lilleifors test and for a nonrandom association between residuals and the estimates by visual inspection of the residual versus estimate plot. All ICAM-1 median fluorescence results were reported as least-square mean ± standard error of the mean. A $P < 0.05$ was considered to be statistically significant.

## RESULTS

### Isolation of Conjunctival Mast Cells and Epithelial Cells

After the first (one-step) Percoll gradient, recoveries of mast cells from the pellet ranged from 0.7 to $1.0 \times 10^6$ total cells/g tissue digested (with mast cell purities of 5%–10%). After the second (two-step) Percoll gradient, recoveries from the interface ranged from 2 to $5 \times 10^4$ mast cells/g tissue digested (>95% purity). Contaminating cells were erythrocytes and mononuclear cells, and the viability of the cell preparations, as determined by trypan blue exclusion, was routinely >98%. The purification procedure did not cause gross alterations in mast cell morphology, as indicated by electron microscopy (Fig. 1). The effects of CaI and anti-IgE (two doses) challenge on mast cell histamine release were determined ($n = 4$). As previously demonstrated in our studies using semipurified mast cells,[6] CaI at a dose of 10 μg/ml resulted in histamine release (61.1% ± 7.4% of total histamine). Challenge of the mast cells with anti-IgE resulted in histamine release in a dose-dependent manner (1 μg/ml, 20.9% ± 4.0%; 10 μg/ml, 31.0% ± 4.0%). Spontaneous histamine release was 4.0% ± 0.5% of the total, and the histamine per cell was calculated to be 2.2 ± 0.2 pg/cell. Recovery of epithelial cells from the top layer of the Percoll gradient ranged from 1.0 to $2.0 \times 10^6$ total cells/g tissue digested. Viability of these cells was routinely 60% to 80% by trypan blue exclusion. This viability is not unusual because any dead or damaged cells are also expected to remain on the top of the single-density Percoll gradient. Nonviable cells do not adhere when cultured and are removed by aspiration from the monolayer. Conjunctival epithelial cells harvested from monolayer cultures were greater than 98% viable as determined by trypan blue exclusion. Purity of the cultured epithelial cells was routinely greater than 95% as determined by cytokeratin staining using FACS analysis (data not shown).

### Conjunctival Mast Cell Tumor Necrosis Factor-α

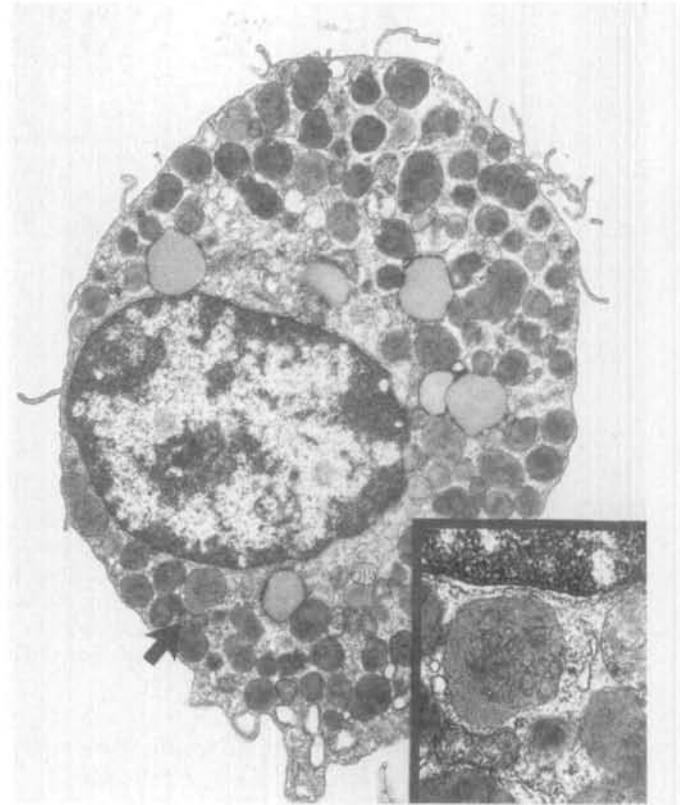In our first experiments, we examined RNA obtained from purified conjunctival mast cells for TNFα expression. Southern



**FIGURE 1.** Electron micrograph of a purified conjunctival mast cell. Typical mast cell granules with electron-dense material and scrolls (*arrow* points to granule in the *inset*) are seen. (Main micrograph magnification, 11,000×; inset magnification, 27,000×).

blots of the RT–PCR products from the purified conjunctival mast cell RNA are depicted in Figure 2. As an indicator of the amount of total cellular RNA processed, the cDNA was also probed for G3PDH, a housekeeping gene with relatively stable expression. RNA isolated from purified conjunctival mast cells was found to contain mRNA specific for TNFα.

In our next set of experiments, we examined mast cells for intracellular cytokine protein content. In these experiments, to assure that we were measuring TNFα only from mast cells, two-color FACS analysis was performed (see Methods). In this procedure, mast cells in the semipure conjunctival cell preparation were identified by their unique mediator, tryptase. Figure 3 is a representative histogram demonstrating the population of tryptase-positive cells that were analyzed for mast cell TNFα staining. Single-color overlay histograms for cytokine staining were generated by gating on the tryptase-positive cells. Intense staining for TNFα was observed in tryptase-positive conjunctival cells (Fig. 4). The percentage of cells staining brighter than the control was calculated by using the number of events in the gate indicated using the following: (Cytokine events in gate − control events in gate/total events) × 100.

Although virtually all mast cells (96%) in the conjunctival cell preparation demonstrated TNFα intracellular staining, the histograms sometimes contained small populations of cells that stained less positively, resulting in separate small peaks (data not shown).
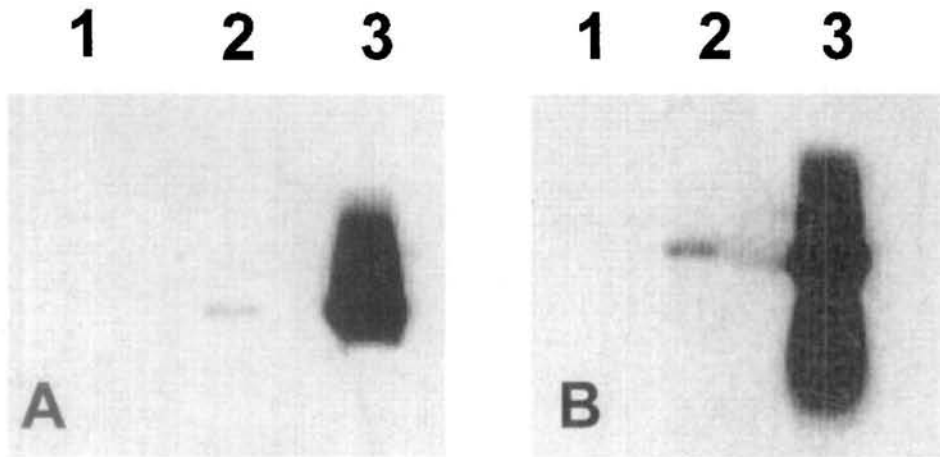
**FIGURE 2.** Southern blots of reverse transcription–polymerase chain reaction (RT–PCR) products from purified conjunctival mast cell RNA. The blots were probed for either (**A**) TNFα or (**B**) glyceraldehyde-3-phosphate dehydrogenase (G3PDH)-specific DNA. The probes were labeled with horseradish peroxidase using luminol as the substrate, and the films were exposed for 10 minutes. In each blot, the samples loaded in the lanes (from *left* to *right*) were (*1*) negative control, (*2*) RT–PCR product from conjunctival mast cell preparation, and (*3*) positive control (commercially prepared TNFα cDNA).

## FACS Analysis of Intercellular Adhesion Molecule 1 Expression on Conjunctival Epithelial Cells

The various treatment effects on conjunctival epithelial cell ICAM-1 expression were compared by FACS analysis. Figure 5 shows representative histograms of anti-ICAM-1-stained cells (markers indicate median fluorescence values before subtraction of isotype control values). There was detectable baseline expression of epithelial cell ICAM-1 (106.0 ± 13.6 MFU, $n = 5$), which was significantly upregulated (Fig. 5A) by incubation of the epithelial monolayers with recombinant TNFα (205.8 ± 13.6 MFU, $P = 0.0001$, $n = 5$). Incubation of epithelial cells

with CaI-conditioned, mast cell supernatants (Fig. 5B) caused an increase in ICAM-1 expression (166.0 ± 13.6 MFU, $P = 0.0071$, $n = 5$).

To verify that TNFα was inducing the ICAM-1 upregulation caused by incubation with mast cell-conditioned supernatants, neutralizing experiments were performed using specific anti-TNFα polyclonal antibody. Incubation of TNFα-neutralizing antibody with recombinant TNFα (Fig. 5C) resulted in a significant decrease in epithelial cell ICAM-1 upregulation (146.0 ± 22.9 MFU, $P = 0.0404$, $n = 3$). In a preliminary
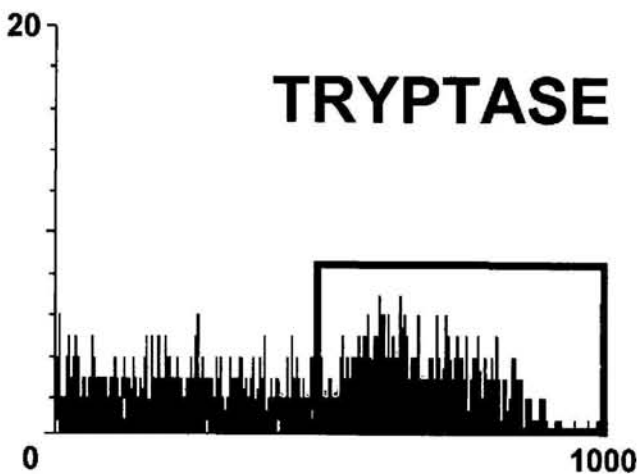


**FIGURE 3.** A representative histogram showing anti-tryptase-fluorescein isothiocyanate (FITC) (*black*) versus isotype antibody-FITC control (*gray*) staining of fixed and permeabilized semipurified human conjunctival mast cells. The *y* axis represents the number of events, and the *x* axis represents the fluorescence intensity. The *boxed area* represents the population of tryptase-positive cells that were analyzed for mast cell TNFα-phycoerythrin (PE) staining in the double-stained preparations.
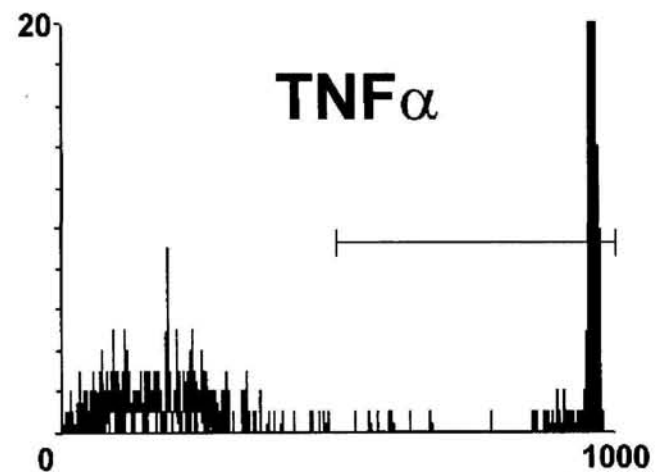


**FIGURE 4.** A representative overlay histogram of phycoerythrin (PE) staining (from four experiments) generated by gating on the tryptase-positive cells and showing anti-TNFα-PE (*black*) versus isotype antibody–PE control staining (*gray*) of fixed and permeabilized conjunctival mast cells (96% positive). The *y* axis represents the number of events, and the *x* axis represents the fluorescence intensity. The percentage of cells staining brighter than the control was calculated by using the number of events in the gate indicated using the following: (cytokine events in gate − control events in gate/total events) × 100.
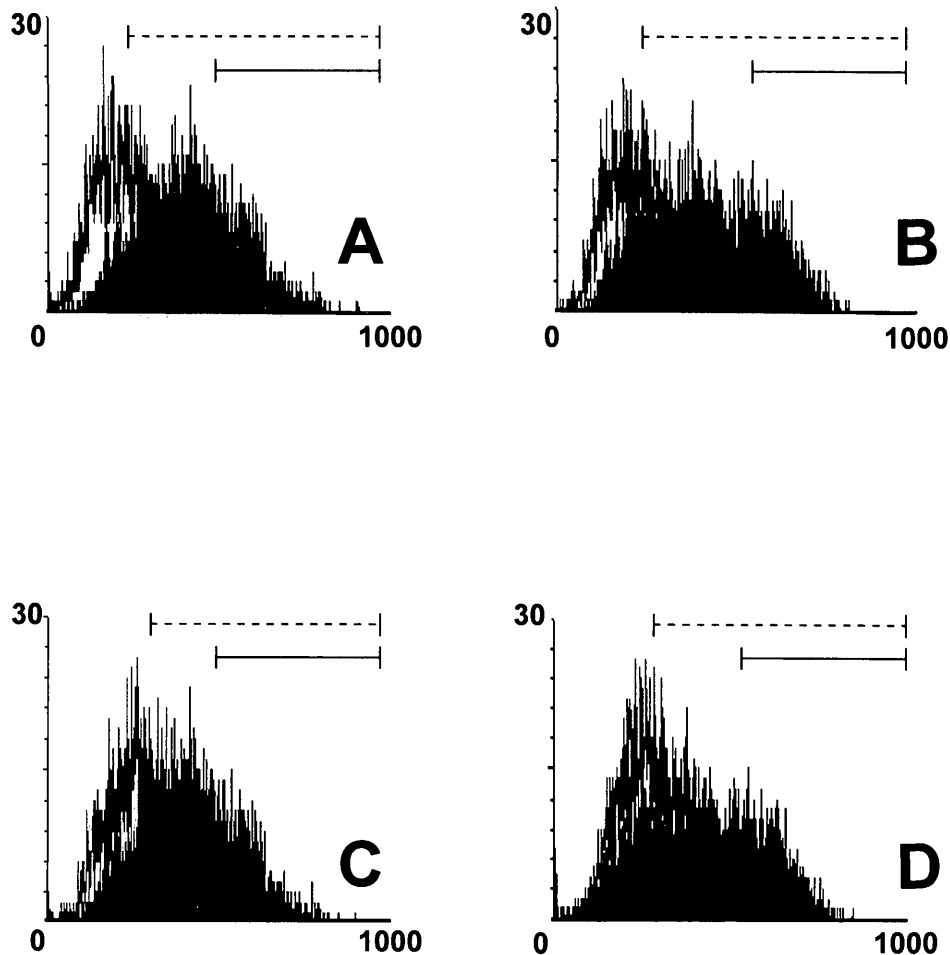
**FIGURE 5.**    Representative histograms showing anti-intercellular adhesion molecule 1 (IC-AM)-phycoerythrin staining of conjunctival epithelial cells exposed to the following treatments: (A) Recombinant TNFα (*black*) compared with constitutive (*gray*) (*n* = 5). (B) Mast cell-conditioned supernatant (*black*) compared with constitutive (*gray*) (*n* = 5). (C) Recombinant TNFα (*black*) compared with recombinant TNFα incubated with anti-TNFα neutralizing antibody (*gray*) (*n* = 3). (D) Mast cell-conditioned supernatant (*black*) compared with mast cell-conditioned supernatant incubated with anti-TNFα neutralizing antibody (*gray*) (*n* = 3). The *y* axis represents the number of events, and the *x* axis represents the fluorescence intensity. Histograms were gated for median fluorescence. The *dashed gate* (*top*) indicates the median fluorescence of the *gray* histogram; the *solid gate* (*bottom*) indicates the median fluorescence of the *black* histogram.

experiment, two doses (0.02 μg/ml and 0.2 μg/ml) of TNFα-neutralizing antibody were incubated with the mast cell-conditioned supernatants, and a dose-dependent decrease in ICAM-1 upregulation was observed. Subsequent experiments were conducted using only the dose of 0.2 μg/ml. Incubation of mast cell-conditioned supernatants with this dose of TNFα-neutralizing antibody (Fig. 5D) resulted in a significant decrease in epithelial cell ICAM-1 upregulation (91.2 ± 18.5 MFU, *P* = 0.0054, *n* = 3).

It was found that incubation with CaI alone (control) resulted in a slight upregulation of ICAM-1 expression (145.1 ± 15.7 MFU). This upregulation, however, was unaffected by anti-TNFα neutralizing antibody (130.1 ± 22.9 MFU). TNFα-neutralizing antibody had no effect on baseline epithelial cell ICAM-1 expression, suggesting that endogenous release of TNFα was not responsible for baseline epithelial cell ICAM-1 expression. Actinomycin D treatment resulted in a marked decrease in baseline epithelial cell ICAM-1 expression mea-

sured at 24 hours, suggesting that there is some upregulation initiated during the 24-hour incubation period by the monolayer itself that is likely not a result of endogenous TNFα (data not shown).

## DISCUSSION

This is the first report of methods to obtain highly purified human conjunctival mast cells and epithelial cells in suspension from human cadaveric donor tissue. We believe these methods give us the opportunity to study the molecular mechanisms of mast cell-ocular surface cell interactions in vitro as a model of ocular inflammation and allergy. Mechanical and enzymatic treatment of human tissues to isolate mast cells has been reported in lung,[10-12] skin,[13] intestine,[14] conjunctiva,[6] choroid,[15] and other tissues. The methods to obtain cells vary according to the tissue type, the density of the mast cells

within the tissue, and the sensitivity of the mast cells to the isolation process. Our purification procedure yielded conjunctival mast cells showing typical metachromatic staining (Wright stain and toluidine blue) and typical electron-dense granules when examined by electron microscopy. When isolating and culturing cells, it is always a concern that their mediator-release profile may be altered. Histamine release assays are a standard method used to assess the functional status of isolated mast cells. Histamine released by a secretagogue depends on the type and concentration of secretagogue used and the type of mast cell treated. Normal function of our isolated and purified mast cells was suggested by histamine release in a dose-dependent manner by the secretagogues CaI and anti-IgE. The histamine released by CaI challenge (61%) corresponds with our previously reported[6] response derived from partially purified conjunctival mast cells. The maximum response to anti-IgE challenge (31% at 10 $\mu$g/ml of anti-IgE) was less than previously reported[6] with the partially purified mast cell preparations (48% at 10 $\mu$g/ml of anti-IgE).

A limitation of our mast cell purification method is the relatively low yield of cells. This makes it difficult to characterize the cytokine profile of this cell using more traditional methods. For this reason we used RT-PCR and intracellular FACS analysis[9] to demonstrate and identify conjunctival mast cell TNF$\alpha$. Analysis of purified (>95%) mast cells with RT-PCR demonstrated mRNA specific for TNF$\alpha$. Because of the sensitivity of RT-PCR, it is possible that the message detected could have originated from the small number of other cell types (for example, mononuclear cells) in the mast cell suspensions. This seems unlikely, however, because TNF$\alpha$ protein was not detected in the tryptase-negative and cytokeratin-positive populations analyzed in our intracellular FACS analysis experiments (data not shown). With intracellular FACS analysis, we have demonstrated that conjunctival tissue mast cells contain TNF$\alpha$ protein. The expression of mast cell TNF$\alpha$, as measured by intracellular FACS staining, showed variability in intensity. Interestingly, the histogram patterns for the donor pools sometimes showed small populations of cells within the pool that stained less positively, possibly reflecting a single donor's mast cells. This variability is not surprising and probably reflects the immune status of the various donors. Bradding and coworkers[16] recently reported a sevenfold increase in expression of TNF$\alpha$ in the lung mast cells obtained from biopsies of subjects with asthma over normal subjects.

The vast interactions of cytokines among cells in tissues undergoing inflammatory reactions is found to be more complex with each new cytokine discovered. The list of cytokines found in human mast cells differs with the source of the mast cells. A human mast cell leukemia cell line was found to have mRNA for a number of pro-inflammatory cytokines.[4] Immature mast cells derived from human peripheral blood mononuclear cells spontaneously secrete interleukin-1$\alpha$, interleukin-1$\beta$, interleukin-6, and TNF$\alpha$ in cultures.[17] TNF$\alpha$ is present in human cutaneous mast cells and lung mast cells[5,16] and other inflammatory cells, such as monocytes and Langerhans cells. We believe the finding of TNF$\alpha$ in our isolated and purified conjunctival mast cells is consistent with the findings in other human mast cells.

The release of preformed and newly synthesized (produced and synthesized in response to stimulation) TNF$\alpha$ has been reported in mice[18] and human dermal mast cells.[19] Storage of preformed TNF$\alpha$ in mast cell granules is important because it could implicate the mast cell as the first source of

this inflammatory cytokine in response to allergen challenge. TNF$\alpha$ has a wide range of biologic effects alone and in concert with other cytokines in vivo and in vitro. Fibroblast proliferation increases in response to low concentrations of TNF$\alpha$ and is inhibited by high concentrations of TNF$\alpha$.[20] Additionally, TNF$\alpha$ has been shown to stimulate eotaxin (a specific eosinophil chemokine) in a human epithelial cell line.[21] Our results demonstrate not only that mast cell-derived TNF$\alpha$ in the conjunctiva is preformed (because it is detected in unstimulated cells), but that it will upregulate ICAM-1 on conjunctival epithelial cells. Although it is possible that the in vitro response of cultured epithelial cells to cytokines is altered with respect to epithelial cells in their natural environment, the response we observed is consistent with the effect of TNF$\alpha$ on other epithelial cells.[22,23] ICAM-1 may participate in the recruitment of inflammatory cells during allergic inflammation, and it has been shown to be an important marker for inflammation in the conjunctiva.[24]

In the most severe form of ocular allergy, atopic keratoconjunctivitis, a significant immunopathologic reaction, occurs in the conjunctiva. Biopsied tissues demonstrate increases in mast cells, goblet cells, eosinophils, the helper to suppressor T-cell ratio, and collagen.[2] Our goal in purifying conjunctival mast cells is to study whether activation of these cells can provide the necessary cytokine signaling to cause changes in conjunctival epithelium, fibroblasts, corneal epithelial cells, and keratinocytes. Our findings suggest that mast cell-derived cytokines prime conjunctival epithelial cells to affect cellular adhesion and potentially to recruit and activate other cells, such as the eosinophil, fibroblast, and keratinocyte. All these cells are active participants in allergic conjunctival inflammation.

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## References

1. Foster CS, Cologne M. Atopic keraconjunctivis. *Ophthalmology.* 1990;97:992.
2. Foster CS, Rice B, Dutt J. Immunopathology of atopic keratoconjunctivitis. *Ophthalmology.* 1991;98:1190.
3. Bonini S, Bonini S, Lambiase A, et al. Vernal keratoconjunctivitis: a model of 5q cytokine gene cluster disease. *Int Arch Allergy Immunol.* 1995;107:95-98.
4. Selvan R, Butterfield JH, Krangel MS. Expression of multiple chemokine genes by a human mast cell leukemia. *J Biol Chem.* 1994; 269:13893-13898.
5. Bradding P. Human mast cell cytokines. *Clin Exp Allergy.* 1996; 26:3-19.
6. Miller S, Cook E, Graziano FM, Spellman J, Yanni J. Human conjunctival mast cell responses in vitro to various secretagogues. *Ocul Immunol Inflamm.* 1996;4:39-49.
7. Undem B, Green F, Warner T, Buckner C, Graziano FM. A procedure for the isolation and purification of guinea pig lung mast cells. *J Immunol Methods.* 1985;81:187-197.
8. Gern JE, Vrtis R, Kelly EAB, Dick EC, Busse WW. Rhinovirus produces nonspecific activation of lymphocytes through a monocyte-dependent mechanism. *J Immunol.* 1996;157:1605-1612.
9. Schmid I, Uittenbogaart CH, Giorgi JV. A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification. *Cytometry.* 1991;12:279-285.
10. Church MK, Pao GJK, Holgate ST. Characterization of histamine secretion from mechanically dispersed human lung mast cells:

effects of anti-IgE, calcium ionophore A23187, compound 48/80, and basic peptides. *J Immunol.* 1982;129:2116-2121.

11. Schulman ES, MacGlashan Jr DW, Peters SP, et al. Human lung mast cells: purification and characterization. *J Immunol.* 1982;129:2662-2667.

12. Wardlaw AJ, Cromwell O, Celestino D, et al. Morphological and secretory properties of bronchoalveolar lavage mast cells in respiratory diseases. *Clin Allergy.* 1986;16:163-173.

13. Benyon RC, Lowman MA, Church MK. Human skin mast cells: their dispersion, purification, and secretory characterization. *J Immunol.* 1987;138:861-867.

14. Befus AD, Dyck N, Goodacre R, Bienenstock J. Mast cells from the human intestinal lamina propria: isolation, histochemical subtypes, and functional characterizations. *J Immunol.* 1987;138: 2604-2610.

15. Barney NP, Presley SJ, Yanni JM, Niles AL, Haak-Frendscho M. Isolation of human choroidal mast cells. [ARVO Abstracts.] *Invest Ophthalmol Vis Sci.* 1996;37:S51.

16. Bradding P, Roberts JA, Britten KM, et al. Interleukin-4, -5, and -6 and tumor necrosis factor-α in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol.* 1994;10:471-480.

17. Hamann KJ, Grabbe P, Welker N, Haas B, Algermission BM, Czarnetzki M. Phenotypic evaluation of cultured human mast and basophilic cells and of normal human skin mast cells. *Arch Dermatol Res.* 1994;286:380-385.

18. Gordon JR, Galli SJ. Release of both preformed and newly synthesized tumor necrosis factor alpha (TNF-alpha)/cachectin by mouse mast cells stimulated via the Fc epsilon RI. A mechanism for the sustained action of mast cell-derived TNF-alpha during IgE-dependent biological responses. *J Exp Med.* 1991;174:103-107.

19. Walsh LJ, Trinchieri G, Waldorf HA, Whitaker D, Murphy GF. Human dermal mast cells contain and release tumor necrosis factor α which induces endothelial leukocyte adhesion molecule 1. *Proc Natl Acad Sci USA.* 1991;88:4220-4224.

20. Thorton SC, Por SB, Walsh BJ, Penny R, Breit SN. Interaction of immune and connective tissue cells: I. The effect of lymphokines and monokines on fibroblast growth. *J Leukoc Biol.* 1990;47:312-320.

21. Garcia-Zepeda E, Rothenberg M, Ownby R, Celestin J, Leder P, Luster A. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nat Med.* 1996;2:449-456.

22. Bloemen PG, Van den Tweel MC, Henricks PA, et al. Expression and modulation of adhesion molecules on human bronchial epithelial cells. *Am J Respir Cell Mol Biol.* 1993;9:586-593.

23. Tosi MF, Stark JM, Smith CW, Hamedani A, Gruenert DC, Infeld MD. Induction of ICAM-1 expression on human airway epithelial cells by inflammatory cytokines: effects on neutrophil-epithelial cell adhesion. *Am J Respir Cell Mol Biol.* 1992;7:214-221.

24. Canonica GW, Ciprand,G, Pesce GP, Buscaglia S, Paolieri F, Bagnasco M. ICAM-1 on epithelial cells in allergic subjects: a hallmark of allergic inflammation. *Int Arch Allergy Immunol.* 1995;107:99-102.