In Vitro Interactions between Peripheral Blood Lymphocytes and the Wong-Kilbourne Derivative of Chang Conjunctival Cells

Alexis Chassignol,1,2,3 Emmanuelle Brasnu,1,2,3,4 Christophe Baudouin,1,2,3,4,5 Luisa Riancho,2,5 Jean-Michel Warnet,2,3,4,6 and Françoise Brignole-Baudouin2,3,4,6

PURPOSE. To investigate the interactions between conjunctival cells and peripheral blood lymphocytes (PBLs) in vitro and to analyze the role of benzalkonium chloride (BAC)-induced apoptosis in this model.

METHODS. Wong-Kilbourne derivative (WKD) cells were cocultured in cell-contact cultures or on cell inserts for 1 to 7 days with PBLs, activated or not with phorbol 12-myristate 13-acetate (PMA). Morphologic analyses of cell interactions were performed using membrane stainings (green PKH67 for WKD cells and red PKH26 for PBL), F-actin immunostaining, and scanning electron microscopy. Sub-G1 peak, CD95/Fas, and HLA-DR expression were assessed by flow cytometry (FCM). Specific interactions through the E-cadherin–CD103 complex were studied with FCM and standard immunofluorescence. Five different concentrations of BAC were tested in microplate cytotoxicity assays, to evaluate cytotoxic effects on cell viability and apoptosis.

RESULTS. WKD/PBL coculture allowed obvious cell interactions, as shown through plasma membrane exchanges. Direct-contact coculture potentiated the BAC cytotoxic effects and increased HLA-DR and CD95/Fas expression on WKD cells. Trichostatin A-pretreated WKD/PBL coculture induced a slight increase in CD103 expression on PBLs. Moreover, the presence of PBLs during the recovery period after WKD cell BAC stimulation reduced WKD cell apoptosis.

CONCLUSIONS. These results suggest that the in vitro interaction of PBLs with WKD cells participates in BAC-induced epithelial toxicity regulation, probably through cell membrane contacts. (Invest Ophthalmol Vis Sci. 2012;53:1492–1498) DOI:10.1167/iovs.11-7708

Cooperative interactions between conjunctival epithelial cells and associated lymphoid tissue composed of dendritic cells, macrophages, and T lymphocytes, actively regulate the inflammatory processes at the ocular surface.1 Epithelial cells may participate in ocular surface inflammatory reactions, as earlier studies on various ocular surface diseases have demonstrated that conjunctival epithelial cells may overexpress human leukocyte antigen DR (HLA-DR), with a profile depending on Th1/Th2 activation and with a Th17 differentiation profile through a dendritic cell–mediated pathway.2–5 Patients with severe allergic conjunctivitis, dry eye disease, or glaucoma treated over the long term may therefore exhibit measurable inflammatory profiles that can be monitored to evaluate novel treatments such as topical cyclosporin or reproduced in vitro models.6

Likewise, in the past few years, the deleterious effects of preservatives such as benzalkonium chloride (BAC) in eye drops administered over the long term have been greatly emphasized, with a clear demonstration of its toxic, proapoptotic, and proinflammatory effects on the ocular surface.7 Our team has repeatedly assessed the relationship between BAC, apoptosis, and the inflammatory processes in epithelial cells in various epithelial cell lines.8–11 Cell line cultures allow quick and inexpensive cell growth in comparison with primary cultures. Moreover, reproducibility and stability are two major advantages of using cell lines. The Wong-Kilbourne derivative (WKD) of the Chang conjunctival cell line is the first established and continuous untransfected epithelial cell line from normal human conjunctiva and has been widely used in previous experiments, particularly for the assessment of the toxicity of preservatives and preserved antiglaucoma eye drops.

The conjunctival mucosa is also part of the mucosal immune system.12–14 Its role in the immune system is supported by the presence of conjunctiva-associated lymphoid tissue (CALT) composed of aggregated lymphatic cells, as CALT participates in the induction of antigen-specific IgA production by B cells against exogenous antigen in the conjunctiva.15 However, this immune counterpart of the conjunctiva has not been considered in previous in vitro studies conducted on conjunctival cells alone. Previous experimental models involving cocultures of lymphocytes and corneal cells, including a part of the conjunctival immune system, have been used to study such relations.16,17 Iwata et al.18 used peripheral blood lymphocytes (PBLs) cocultured with human corneal epithelial (HCE) cells to determine the role played by the interactions between epithelial and allogeneic lymphocytes in the lymphocytic activation process. They demonstrated that IFN-γ-treated HLA class II-bearing HCE cells stimulated freshly isolated allogeneic lymphocytes. Moreover, Gomes et al.19 described a new, interesting coculture experiment in primary cultures of human corneal or conjunctival epithelial cells with peripheral blood lymphocytes (PBLs). They studied human mucosal lymphocyte antigen
(HML)-1/CD103 expression on PBLs induced by direct and indirect interactions with ocular surface epithelial cells, showing that this HML-1 expression on CD8^+ lymphocytes did not correlate with PBL activation status.

The purpose of this study was to investigate the in vitro epithelial cell–lymphocyte interactions and then to evaluate the toxicity profile of BAC on epithelial cells with the presence of activated and inactivated immune cells. Therefore, we assessed the morphologic, inflammatory, apoptotic, and toxicologic responses of both cell types in this coculture model.

METHODS
Conjunctival Cell Line and PBLs
The WKD of Chang conjunctival cells (clone 1-5c-i; ATCC, Manassas, VA, certified cell line [tCCl] 20.2) were cultured in standard conditions. At confluence, the cells were removed by gentle trypsin incubation, seeded into six-well cell culture plates for cytometric analyses, and seeded on slides (Laboratory-Tek II chambered coverslip; Nunc International, Naperville, IL) for standard immunofluorescence (IF) and confocal microscopy. Subconfluent cells (culture surface covering nearly 70%) were exposed to the different BAC concentrations. In partnership with the French Blood Agency (FBA), lymphocytes were obtained from peripheral blood of healthy donors. Only one donor was needed for each phase of coculture. In the end, 15 donors were used for all the experiments. The FBA performed plateletpheresis on the donors and the remains of this procedure (cytapheresis concentrate) were used to separate the PBLs from red blood cells, by centrifugation at 400g for 30 minutes on a single-density gradient (Ficoll; Sigma-Aldrich, St. Louis, MO) to isolate the mononuclear cells. PBLs were washed in phosphate-buffered saline (PBS), centrifuged at 300g for 15 minutes, washed again in PBS, and centrifuged at 200g for 10 minutes. The PBLs were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% fetal bovine serum (FBS) and cultured under standard conditions for 12 hours. Then the PBLs (1 mL of 10^6 cells/mL) were cocultured with confluent epithelial WKD cells, either in direct contact with the epithelial WKD cells or separated from the epithelial cells by a cell culture insert (Transwell translucent PET membrane, 0.4-μm pores; BD Falcon, BD Biosciences, Franklin Lakes, NJ) allowing free interchange of culture medium between the two compartments.

Cell Treatments
PBLs were incubated with 2.5 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 250 ng/mL of ionomycin (Sigma-Aldrich) at 37°C for 2 hours. After stimulation, the cells were washed three times with RPMI. WKD cells were treated with trichostatin A at 0.33 μM (TrichostatinA Ready-Made Solution, 5 mM in DMSO [0.2 μM-filtered], from Streptomyces sp; Sigma-Aldrich) for 24 hours in standard conditions in complete culture medium supplemented with 10% FBS, to induce E-cadherin expression on WKD cells.20 WKD cells were exposed to five different concentrations of BAC (5 × 10^-3%, 10^-2%, 5 × 10^-3%, 10^-3%, and 5 × 10^-4%) or PBS for 15 minutes followed by 24 hours of cell recovery in complete culture medium for evaluation of cell viability and the cytotoxic effect of BAC in the coculture model.21 These BAC concentrations are equivalent to or lower than the concentrations of most available eye drops.

Flow Cytometry
Flow cytometry was performed with a flow cytometer (Cytomics FC500-CXP; Beckman Coulter, Miami, FL). The class II antigen HLA-DR (clone Immu-357, mouse fluorescein isothiocyanate [FITC]-conjugated; Beckman Coulter), the apoptosis-related receptor CD95/Fas (clone UB2, mouse purified; Serotec, MorphoSys AG, Martinsried/Planegg, Germany) were tested on WKD cells. CD103/HML-1 (clone 2G5, mouse FITC-conjugated; Coulter Corp.) was tested on PBLs. FITC-conjugated goat anti-mouse and phycoerythrin (PE)-conjugated rabbit anti-mouse antibodies were obtained from Dako (Glostrup, Denmark). Alteration of DNA content was measured through sub-G1, peak flow cytometric analysis on a FL1-fluorescence histogram to characterize the late apoptosis process by a previously described method.22 After 15 minutes of BAC treatment followed by 24 hours of cell recovery in complete medium, the cells were collected by using EDTA and fixed with 0.5% paraformaldehyde (PFA) fixative in PBS at 4°C for 24 hours. After the 24-hour fixation, the samples were washed with cold PBS, permeabilized in 0.1% saponin, stained with 50 μg/mL propidium iodide (Interchim, Montluçon, France), and analyzed on the flow cytometer.

Microplate Cytosensor
Microplate cytosensor was performed on a microplate cytometer (Safire; Tecan Instruments, Lyon, France). Hoechst 33342 dye was used to evaluate WKD cell apoptosis. The cells were exposed for 30 minutes to a Hoechst 33342 solution at a concentration of 10 μg/mL containing 1 μL of propidium iodide (1 mg/mL in water). Neutral red (NR) dye (Fluka, Ronkonkoma, NY) was used to evaluate membrane integrity, correlated with cell viability, used at a concentration of 50 μg/mL. Fifty microliters per well of neutral red solution was added to living cells. After a 4-hour incubation time at 37°C in moist atmosphere with 5% CO₂, the neutral red fluorescence signal was measured. Each drug was tested in six wells (50 μL per well), and each experiment was performed in triplicate. The results were obtained in fluorescence units and expressed as a percentage of the control PBS. Hoechst 33342 results were expressed as a ratio of the results of this assay to the results of the NR assay, to correlate them with cell viability.

Cell Morphology Analyses
Standard IF stainings, using F-actin detection (Alexa Fluor 488 phalloidin; Molecular Probes, Leiden, The Netherlands), the lipophilic green-emitting dye PKH67 (Green Fluorescent Cell Linker Kit; Sigma-Aldrich) for WKD cells, and the lipophilic red-emitting dye PKH26 (Red Fluorescent Cell Linker Kit; Sigma-Aldrich) for PBLs, were performed and examined under an epifluorescence microscope (E800, PCM 2000; Nikon, Tokyo, Japan), to assess morphologic patterns. Cell interactions were also examined by scanning electron microscope (SEM). The cells were fixed in 2.5% glutaraldehyde (Electron Microscope Sciences, Hatfield, PA) for 45 minutes, then in 2% reduced osmium OsO₄ (Electron Microscopy Sciences) for 45 minutes. The cells were dehydrated in ethanol, cleared in propylene oxide, and embedded in hexamethylene disilane (Sigma-Aldrich). Then, the cells were sputter coated with gold palladium by ion sputtering (JEOL Fine Coat, JFC-1100; JEOL, Tokyo, Japan) and examined with a scanning electron microscope (JEOL 35CF; Hitachi, Tokyo, Japan).

Statistical Analysis
Each experiment was performed in triplicate (Sigma Stat 2.0; SPSS, Chicago, IL). For the microplate cytosensor data, significance was assessed by one-way analysis of variance (ANOVA) followed by the Dunnett test. The flow cytometry data were analyzed by one-way ANOVA followed by the Bonferroni test.

RESULTS
Coculture of WKD/PBLs Resulted in Macropinocytosis in Epithelial Cells
Plasma membrane exchanges between the two cell types were observed after 1 day of coculture in the direct-contact coculture model between epithelial cells and PBLs (Fig. 1A). Controls—either WKD cells alone or WKD cells cocultured with PBLs separated by a 0.4-μm pore translocutent PET membrane...
(Fig. 1B)—showed exclusively green WKD cells, without any transfer of red staining by PBL to WKD cells. An obvious integration of lymphocyte membranes in epithelial cell membrane rapidly occurred, in a time-dependent manner. A peak was obtained after 90 minutes of coculture (Fig. 1C). These close connections between the two cell types, resembling cell membrane fusion, were also observed in SEM (Fig. 1D), and this phenomenon seemed to be similar to pinocytosis. Lymphocytes showed cytoplasmic expansions after phalloidin staining and fluorescence microscopy analysis (Fig. 1E).

**Interactions between WKD Cells and PBLs**

**Increased Expression of Inflammatory Markers**

HLA-DR expression increased on WKD after 7 days of direct-contact coculture with nonactivated PBLs, and after 7 days of noncontact coculture with activated PBLs (Fig. 2). CD95/Fas antigen was expressed on WKD cells after 1 day of direct-contact coculture with activated PBLs. Furthermore, WKD shrinkage
increased in a similar manner after 1 or 7 days of coculture, as shown with phalloidin and propidium iodide staining (data not shown).

**WKD Cell Interactions with PBLs Switched PBL to an IEL Phenotype**

Next, we examined whether PBLs would express the intraepithelial lymphocyte (IEL) phenotype marker CD103 after coculture with WKD cells. In our experiments, no CD103 expression on PBLs was obtained after WKD/PBL coculture, whereas a previous study had shown CD103 expression on PBLs after coculture with primary cultured conjunctival cells. Although E-cadherin, the CD103 ligand on epithelial cells, was found on conjunctival cells in primary culture, in the present experiments, E-cadherin expression was not observed constitutively or after coculture with PBLs by FCM and standard IF.

**DISCUSSION**

In these series of experiments in an original model of lymphocyte/conjunctival epithelial cell coculture, we observed close in vitro interactions between WKD cells and PBLs. These interactions were related to membrane exchanges, cell membrane fusion, and endocytosis. In our model, endocytosis of large PBL membrane fragments did not undergo fusion with lysosomes, but the internalized material was recycled back to the cell membrane in less than 24 hours. This phenomenon was thus similar to macropinocytosis. In mucosal tissues, macropinocytosis has been reported to be a support for the scavenging activity of resident macrophages, and in epithelial cells, it has been shown that EGF-induced macropinocytosis may capture and internalize the material that is rapidly recycled back to the cell surface. Moreover, by SEM, we observed that close in vitro interactions between the two cell types also led to cell membrane fusion.

In the present study, HLA-DR expression increased on WKD cells after 7 days of coculture either in direct contact with nonactivated PBLs or in contact with activated PBLs. The apoptosis-related marker CD95/Fas was expressed on WKD cells after only 1 day of direct-contact coculture with activated PBLs. According to these results, it can be hypothesized that in the presence of PBLs, cell activation and apoptosis could be mediated by the same stimuli and transduction pathways, with close cell interactions, through direct cell contact or through soluble mediators. Nevertheless, an allogeneic stimulation can also be hypothesized. Gomes et al. (IOVS 1995;36:ARVO Abstract S840) studied the changes induced on surface antigens of nonactivated lymphocytes from the same donors cocultured with corneal epithelial cells from different donors. They observed that any allogeneic response caused by antigen presentation resulted in variable CD25 activation on PBLs. They concluded that using allogeneic lymphocytes for coculture with epithelial cells did not particularly influence the expression of surface antigens on the two cell types.

Nevertheless, trichostatin A pretreatment of WKD cells induced slight E-cadherin expression detected using FCM (Fig. 3A) and standard IF stainings (Fig. 3B), slightly increasing CD103 expression on PBLs (Fig. 3C), the mark of transformation of the PBLs into IELs.

**PBL Interaction with WKD Cells Reduced BAC-Induced Epithelial Toxicity**

Subsequently, potential toxicity of BAC on epithelial cells was assessed in the presence or absence of immune cells. WKD cell apoptosis increased in a BAC concentration-dependent manner (from $5 \times 10^{-4}\%$ to $5 \times 10^{-2}\%$), whether or not PBLs were present during the recovery period. Furthermore, for the lowest concentrations of BAC ($<5 \times 10^{-5}\%$), the presence of PBLs in coculture during the recovery time increased apoptosis after 1 day of direct contact as a potentiation of the apoptotic effect of BAC. Conversely, for higher concentrations of BAC ($>5 \times 10^{-5}\%$), the presence of PBLs in direct-contact coculture during the 24-hour recovery period induced a significant decrease in apoptosis of WKD cells (Fig. 4).
The mucosal immune system traditionally includes IELs that are predominantly a specialized subpopulation of CD8+ T cells with an αβ-positive T-cell receptor. More than 90% of these IELs express HML-1/CD103 versus only 2% of PBLs. HML-1 is an adhesion molecule belonging to the integrin family, composed of the β7 subunit in association with the αE subunit. It mediates specific interactions between IELs and mucosal epithelial cells via a tissue-restricted adhesion molecule called E-cadherin. Gomes et al. found expression of HML-1/CD103 on PBLs after 7 days of coculture, with epithelial cells in primary culture indicating a transformation of PBLs into IELs. Seven days seemed to be the optimal incubation time for HML-1 expression on activated lymphocytes. In our study, HML-1 expression was not induced on PBLs after 1 to 7 days of coculture with WKD cells. The main difference between epithelial cells in primary culture and WKD cells is the loss of E-cadherin expression on the WKD cells’ surface. Using trichostatin A, a histone deacetylase (HDAC) inhibitor, we observed weak E-cadherin expression on WKD cells. Consequently, slight CD103/HML-1 expression was observed on PBLs after 1 to 7 days of coculture with E-cadherin-positive WKD cells. Further experiments are therefore needed to assess the ability of PBLs to develop an IEL phenotype after coculture with WKD.

Other studies have shown the proapoptotic and proinflammatory effects of BAC on WKD cells with a BAC concentration-dependent increase in vitro, consistent with ex vivo observations of the conjunctiva of glaucoma patients treated over the long term with preserved eye drops. Its ocular cytotoxicity has been demonstrated in many in vivo and in vitro single-cell models. However, human conjunctival epithelium is composed not only of conjunctival epithelial cells but includes other cell types such as immune and goblet cells that are in close relation with blood vessels and extracellular lymphocytes. In vitro interactions between both immune and epithelial cell types have already been studied in other systems, but no information is currently available on the effects of BAC on complex systems where immune cells and epithelial cells interact. In the present study, PBLs tended to show a protective effect on the WKD cells against BAC for the highest BAC concentrations (≥5 × 10^-7 M), concentrations where WKD apoptosis was at its maximum. Conversely, with lower concentrations of BAC, PBLs seemed to have proapoptotic effects on the WKD cells. Previous studies have already shown a neuroprotective role of T cells in proapoptotic situations through neuroprotective molecules, consistent with the concept of protective autoimmunity. On the other hand, our team has already demonstrated the proinflammatory role of BAC on epithelial cells.

In conclusion, this study is a first step toward a better understanding of the in vitro relationships and interactions between conjunctival epithelial cells and lymphocytes. Close connections and cell interactions through soluble mediators or direct cell contact have been observed, with proinflammatory and proapoptotic effects illustrated by HLA-DR and CD95/Fas expression or the sub-G1 profile. These phenomena may not be entirely explained by an allogeneic reaction between cell lines from one donor and immune cells from another. E-cadherin expression was observed on WKD cells, with a trend toward CD103/HML-1 expression on PBLs, demonstrating a switch to an IEL phenotype. Moreover, a dual protective or proapoptotic effect of immune cells on WKD cells depending on the BAC concentration was found, opening up very promising perspectives on the effects of xenobiotics on the ocular surface and possible interactions between immune cells, epithelial cells,
and environmental stresses. This evidence leads us to believe that the immune part of the conjunctiva may play a major role in the regulation of preservative-induced epithelial toxicity observed every day in clinical practice in patients treated with preserved eye drops. The next step could be to use a similar approach to further explore the interactions between conjunctival cell lines and specific immune populations such as lymphocyte subpopulations or dendritic cells in an in vitro coculture system, using established cell lines of both epithelial and monocyte/macrophage lineages, to respond to the issues of primary cultures and variability intrinsic to individual donors and to complete experiments assessing cytokine-induced or Fas-mediated toxicity.

References


