Area and Depth of Surfactant-Induced Corneal Injury Correlates with Cell Death

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PURPOSE. In previous studies in which in vivo confocal microscopy (CM) was used, quantifiable differences were identified in the corneal epithelium and stroma for surfactants producing different degrees of ocular irritation. In the present study, in vivo confocal microscopy was used to determine area and depth of the initial corneal changes, and the correlation of the data to cell death was characterized by ex vivo live-dead assay.

METHODS. In four groups of rabbits (12 animals each), 10 μl surfactants known to produce slight, mild, moderate, or severe irritation was applied to the central cornea of one eye; 4 untreated rabbits served as controls. Measurements of group total mean epithelial thickness, epithelial cell area, and depth of keratocyte loss in four corneal regions were made by in vivo CM in 6 rabbits of each group and in 4 control animals at 3 hours and in the remaining rabbits at 3 hours and 1 day. Corneas were then removed and fixed for conventional histologic examination (two eyes/treatment/group), or regions were excised and placed in culture media containing 2 μM calcein-acetoxymethyl ester (calcein-AM) and 4 μM ethidium homodimer. Using laser scanning CM, the number of dead epithelial or stromal cells in a 300 X 300 X 170 μm (in the x, y, and z axes, respectively) volume of the cornea was determined.

RESULTS. Confocal microscopy showed that application of the slight irritant resulted in decreased epithelial thickness at 3 hours (41.2 ± 2.6 μm in controls versus 46.3 ± 3 μm in control eyes; n = 6 and 4, respectively) and a significant decrease (P < 0.001) in epithelial cell size (630 ± 203 μm² versus 1427.2 ± 90.7 μm²). On day 1, mild, moderate, and severe irritants caused complete loss of epithelium and disappearance of keratocytes to a depth of 30.8 ± 10.7 μm, 47.2 ± 10.4 μm, and 764.6 ± 159.6 μm (n = 6, 5, and 6), respectively. At 3 hours, live-dead assay detected more dead epithelial cells as a percentage of total surface cells (49.2 ± 4.5% in slightly irritated eyes versus 20.9 ± 3.2% in control eyes), significantly correlating with the measurement by in vivo CM of average epithelial cell size in each eye (r = -0.96; P < 0.005). On day 1, mild and moderate irritants showed increasing stromal cell death from 9.8 ± 16.2 cells to 36.4 ± 17.7 cells, which significantly correlated with the depth of stromal injury determined by in vivo CM (r = 0.79; P < 0.0001). No surviving keratocytes were detected in severely irritated eyes.

CONCLUSIONS. The data support the hypothesis that differences in surfactant-induced ocular irritation are directly related to area and depth of acute corneal injury. (Invest Ophthalmol Vis Sci. 1998; 39:922-936)
the difficulty in comparing and correlating objective, quantitative in vitro data with inherently subjective scoring of macroscopic changes in animal tests. A mechanistic understanding of ocular irritation in the context of these standard in vivo tests is needed. Ideally, this would include histopathologic and biochemical data that could be applied to the development and validation of mechanistically based in vitro tests.

Unfortunately, making objective, quantitative assessments in these standard tests is difficult. For example, in the Draize test, test materials in a 100-μl dose are placed in the inferior cul-de-sac of the rabbit eye, and the lids are briefly held closed. Because the lower cul-de-sac holds only 30 μl, material may be lost; and different regions of the eye may receive varying doses of test material. This may result in considerable variation in the distribution of test material across the ocular surface and may lead to variations in injury and responses occurring within the same eye and among eyes. In the LVET, only 10 μl test material is directly applied to the central cornea without forced lid closure. Although the LVET shows better correlation with human exposure and is less stressful to the animal, uneven exposure of the ocular surface in the same eye remains a potential problem. These variations in test material distribution across the ocular surface cause two basic problems in quantitative assessment of ocular irritation. First, sampling of the eye for histologic or biochemical analyses requires evaluation of the whole eye or multiple regions of the same eye to establish the overall area and depth of injury. Second, variations in exposure among eyes, not considering variation in individual animal responses, makes the correlation of initial histologic or biochemical changes with later outcomes or responses difficult and may require the use of very large groups of animals to establish significant correlations.

Our laboratory has been interested in defining the pathobiologic nature of ocular irritation in the context of standard animal tests to validate in vitro alternatives for determining ocular irritation. Previously, we showed that in vivo confocal microscopy (CM) can be used for objective assessment of damage to the corneal epithelium, stroma, and endothelium after the application of slightly and severely irritating surfactants, without killing the animal. Furthermore, sampling multiple regions of the ocular surface provides an estimate of the area and depth of injury for each eye, which can then be directly correlated to the subjective Draize and LVET findings. Recently, we have shown that a range of surfactants that cause differing degrees of ocular irritation produce quantitative differences in the area and depth of initial injury to the cornea in relation to their potential to cause irritation—that is, slight surfactants produce less irritation than do mild ones, and so on, with severe surfactants producing the most irritation (that is, slight < mild < moderate < severe). Based on these and other findings, we have proposed that the degree of corneal irritation is dependent on the initial area and depth of injury and that area and depth of injury subsequently determine the response and final outcome of exposure to irritating surfactants.

In the present study, we extended these observations to show that initial area and depth of corneal injury, measured by in vivo CM correlates with surfactant-induced death of the corneal epithelium, keratocytes, and endothelium measured by an ex vivo live–dead assay (L-D; Molecular Probes, Eugene, OR) and that the magnitude of cell death correlates with the potential for ocular irritation. Overall, this study supports the concept that area and depth of cell death define the ocular response to irritating surfactants, regardless of the physiochemical process underlying the tissue damage. Furthermore, the data suggest that measurement of area and depth of cell death may be used as a mechanistic correlate in an appropriate in vitro model.

**Materials and Methods**

**Animals**

Fifty-two adult female New Zealand albino rabbits weighing approximately 4 to 6 kg (Myrtle’s; Thompson Station, IN) were used in this study. Animals were identified by ear tags, were individually housed in suspended stainless steel cages, and were maintained in a 12-hour light–dark cycle. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To reduce animal variation, all animals used for L-D analysis (34 rabbits) were shipped from the vendor at the same time and were tested on the same day.

**Test Materials**

Surfactants possessing differing potential to cause irritation, determined by results of historical rabbit Draize eye tests, LVETs, and recent histopathologic and in vivo CM studies were selected. These materials included, sodium lauryl sulfate (5% wt/vol), an innocuous or slightly irritating anionic surfactant; sodium linear alkylbenzene sulfonate (RC₇H₄SO₃Na; r = C₁₂–C₁₃; 35% active), a mildly irritating anionic surfactant; proprietary prototype detergent that causes moderate irritation because of its anionic surfactant; and cetyltrimethylammonium chloride (50% active), a severely irritating cationic surfactant.

**Application of Test Materials**

Test materials were topically applied to one eye of rabbits receiving test surfactants, according to procedures previously described for the LVET. The LVET was used in place of the Draize procedure, because it is less stressful to rabbits and more predictive of the human ocular response. Eyes were evaluated by penlight examination 24 hours before application to ensure the absence of ocular abnormalities. Animals were then divided into five groups: a control group (Control, 4 rabbits), in which no surfactant was applied; a slightly irritated group (Slight, 12 rabbits), in which 5% sodium lauryl sulfate was applied; a mildly irritated group (Mild, 12 rabbits) in which linear alkylbenzene sulfonate was applied; a moderately irritated group (Moderate, 12 rabbits), in which a prototype detergent was applied; and a severely irritated group (Severe, 12 rabbits), in which cetyltrimethylammonium chloride was applied.

To administer the test material, 10 μl surfactant was taken up in a 100-μl, Teflon-tipped syringe, and excess test substance was removed by wiping the syringe tip with a clean paper towel. In the nonanesthetized rabbit, the eyelids were gently held open and the test material placed directly above the center of the cornea in the right eye. Eyelids were then immediately released without forced blinking or manipulation. In the four control rabbits, no test material was applied. All test material application was performed by the same investigator (RDP) to ensure consistent application of doses. In addition, time of application was carefully recorded to ensure that all
subsequent evaluations were made within a range of 15 minutes of the desired observation times.

After application, all animals were assessed for ocular irritation, and results were graded by penlight examination and by in vivo CM at 3 hours. After grading and in vivo CM at 3 hours, six animals each from the slight, mild, moderate, and severe test groups and the four control animals were killed by intravenous injection of sodium pentobarbital (120 mg/kg body weight; Abbott Pharmaceuticals, Farmers Branch, TX), and eyes were obtained for L-D assay (two control eyes and four eyes from each test group) and conventional histologic analysis (two control eyes and two eyes from each test group).

The remaining animals (six rabbits from the four surfactant-treated groups) were then evaluated by penlight examination and in vivo CM at 24 hours after application. After examination, the animals were killed, and eyes were obtained for L-D assay (four eyes from each test group) and conventional histologic study (two eyes from each test group).

**Macrosopic Penlight Examination**

All eyes, treated and control, were examined macroscopically for irritation by the same experienced observer (RDP), using a penlight. Ocular irritation was scored on the basis of standard evaluation methods to assess severity of corneal, iridal, and conjunctival irritation, as originally defined by Draize et al. Specifically, the corneas were evaluated for degree and area of opacity. Each parameter (degree and area) was assigned a grade of 0 to 4. The grades were then multiplied and the product was multiplied by 5 to derive a weighted corneal score that could be as high as 80. The iris was assessed for increased prominence or iridal folds, congestion, swelling, circumscleral injection, hemorrhage, gross tissue destruction, and decreased pugillary reflex. Iridal changes were assigned a grade of 0 to 2, and this grade was multiplied by 5, which provided a maximum weighted score of 10. The conjunctiva was evaluated for degree of redness, swelling, and discharge. Redness and discharge were assigned a score of 0 to 3, and swelling a score of 0 to 4. The weighted conjunctival score was obtained by multiplying the sum of the grades by 2, which provided a maximum weighted score of 20. The maximum total score possible was 110. A material's potential to cause ocular irritation was then determined by assessing the individual animal scores, the maximum average score (the highest mean group score during the study), and days to recovery.

**In Vivo Confocal Microscopy**

After penlight examination, rabbits were anesthetized with 30 mg/kg body weight ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and 3 mg/kg body weight xylazine (Miles, Shawnee Mission, KS), administered intramuscularly. Immediately after induction of anesthesia, treated eyes and the right eyes of control rabbits were viewed using a modified scanning confocal microscope (Tandem Scanning, Reston, VA) with a x24 surface contact objective (0.6 numerical aperture and 1.5-mm working distance) that had a remote-controlled internal lens adjustment to change z-axis focal plane position. The design and use of this microscope and lens system have been described previously. Before scanning, a drop of preservative-free, synthetic tears solution was applied (Lens Plus; Allergan, Irvine, CA) to couple the objective lens tip to the ocular surface. A total of four regions of the cornea were evaluated—central, inferior, nasal, and temporal—by careful movement and positioning of the rabbit, rabbit eye, and microscope to avoid possible accidental damage to the ocular surface induced by the microscope's objective.

In vivo confocal microscopic images were detected using a video camera (VE1000; Dage-MTI, Michigan City, IN) and recorded on video tape (Super VHS BR-S611U; JVC, Elmwood Park, NJ). Three-dimensional (3-D) data were collected by confocal microscopic through-focusing (CMFT) with a constant lens speed of 80 µm/sec, as described previously. During CMFT scanning, the entire cornea, starting at the surface epithelium and extending to the corneal endothelium, was scanned at a constant lens speed, so that single video frames could be correlated later to exact z-axis depths. The entire data set was digitized, stored, and analyzed on a computer workstation with a video image acquisition board (Silicon Graphics, Mountain View, CA). Images were also processed, and the cornea was displayed in three dimensions, by an image processing software program (Analyze; Mayo Medical Ventures, Rochester, MN).

As previously shown, CMFT evaluation of the cornea provides accurate determination of corneal thickness, epithelial thickness, stromal thickness and measurement of corneal light reflectivity by calculating an image intensity depth profile of the CMFT scan. In addition, the CMFT images can be used to locate and accurately determine the depth of unique intraepithelial structures, including surface epithelium, basal lamina, and stromal nerves. In the present study, CMFT intensity profiles were used to measure epithelial and corneal thickness after application. The CMFT video scans were also used to assess mean depth of keratocyte injury (described later). Single images taken from the surface corneal epithelium were also digitized, and the mean surface epithelial cell size (i.e., area) was quantitated according to techniques previously described.

**Ex Vivo Live–Dead Assay**

Immediately after death, the entire cornea with 2 mm of adjacent sclera was removed from the eye, carefully placed epithelial side up on dental wax, and immersed in Dubilbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD) at room temperature. Because of extensive epithelial and stromal injury associated with the severe irritant, 1-day samples from severely irritated eyes were placed endothelial side up to facilitate evaluation of the corneal endothelium. Four corneal blocks (6 mm × 6 mm) corresponding to the central, inferior, nasal, and temporal regions examined by in vivo confocal microscopy were then carefully excised from the cornea, by using a single-edged razor blade (Polysciences, Warrington, PA). Individual tissue blocks were placed in 1 ml of Dubilbecco's modified Eagle's culture medium containing 2 µM calcein-AM and 4 µM ethidium homodimer (Molecular Probes) in 24-well tissue culture plates (Corning Costar, Cambridge, MA). The plates were then placed in a 37°C, 5% CO2 humidified incubator for 30 minutes. Tissues were removed, placed endothelial side down on 25-µm thick, 60-mm diameter, mylar petri dishes (Bachofer, Hamburg, Germany) containing Dubilbecco's modified Eagle's medium, calcein-AM, and ethidium homodimer. Tissues were analyzed ex vivo by scanning laser confocal microscope (Leica; Deerfield, IL) equipped with a fluorescence microscope (Fluorovert; Leica), an argon–krypton laser, and dual photomultiplier tube detect-
FIGURE 1. Light micrographs (A, C) and in vivo confocal microscopy images (B, D) comparing control (A, B) and slightly irritated (C, D) corneas 3 hours after application of slightly irritating surfactant. In vivo micrographs are shown as three-dimensional displays taken from confocal microscopic through-focusing scan through the entire cornea. In the control cornea (B), the surface epithelium (arrows) is composed of highly reflective, large, flat cells, separated from the basal lamina (curved arrow) by nonreflective wing and basal cells. Within the stroma, reflective keratocyte nuclei are shown (arrowheads). Note loss of surface epithelium (C) and smaller surface cell size (D, arrows) after the application of slightly irritating surfactant. Scale bar, 100 μm.

Ex vivo, tissue blocks were initially scanned using conventional epifluorescence with a ×16 water immersion objective (0.5 numerical aperture, 240 μm working distance; Carl Zeiss, Oberkochen, Germany). A single representative area was randomly selected for laser scanning CM and a 3-D data set consisting of a series of images (256 × 256 pixels) extending through ethidium homodimer-stained dead cells to calcein-AM-stained live cells was taken in 2.5- to 5-μm steps. In general, the volume analyzed was approximately 300 μm × 300 μm × 170 μm (in the x, y, and z axes, respectively). The in the x-y area covered by laser scanning CM was comparable with that covered by in vivo CM, but the z-axis depth covered only the anterior third of the cornea. This limit was a function of the objective used in this study, which had a limited working distance. Nevertheless, the distance was adequate to evaluate the depth of cell death after application of slight, mild, and moderate irritants and was only limited in its ability to evaluate the effects of the severe irritant.

Images comprising the 3-D data set were transferred to a workstation (Personal Iris 4D-35G; Silicon Graphics), and maximum-intensity volume renderings were made using the software program mentioned previously (analyze; Mayo Medical Ventures). A series of five y-z (or x-z) slices, 10 pixels wide in the x- or y-axis (approximately 12 μm) and separated by 50 pixels (60 μm), were then taken through the stack of slices. The total number of cells stained with ethidium homodimer in
Normal
At time of death, the anterior chamber was perfused with 1% tissue block. In all cases, L-D examination was completed within time to collect only a single 3-D data set from each analysis had to be completed within this interval. This schedule allowed time to collect only a single 3-D data set from each sample.

Because the time between treating individual animals to be killed was only 40 minutes, data collection for the L-D analysis had to be completed within this interval. This schedule allowed time to collect only a single 3-D data set from each tissue block. In all cases, L-D examination was completed within this time: L-D analysis occurred at approximately 4 hours and 25 hours after application of surfactant; penlight and in vivo confocal examination occurred at approximately 3 hours and 24 hours after application.

Histologic Evaluations
At time of death, the anterior chamber was perfused with 1% glutaraldehyde in 0.2 M phosphate buffer for 10 minutes. Corneas were then excised and fixed in the same solution overnight at 4°C. Three tissue blocks (8 mm X 4 mm) containing the central-nasal, central-temporal, and inferior regions of the cornea with 2 mm of sclera were then taken from each cornea, processed, and embedded in glycolmethacrylate (QB-4; Polysciences). Sections (5 μm thick) were cut using a microtome (Reichert-Jung; Leica), stained with hematoxylin-eosin and viewed using a light microscope (Leitz Diaplan; Leica).

Statistical Analyses
Statistical analyses were performed using commercial software (SigmaStat for Windows Version 1.0, Jandel Scientific, San Rafael, CA; SAS Version 6.11, SAS Institute, Cary, NC; and StatXact, Cytel Software, Cambridge, MA). The Pearson product-moment correlation coefficient was used to evaluate the correlation between two measurements. One-way analysis of variance was performed to determine whether there were differences among multiple test groups, and the Tukey Studentized range test was used to make pairwise comparisons among groups. When standard variance equality assumptions underlying analysis of variance were violated for a given measurement, determined by Bartlett’s test, the statistical analysis was made by analysis of variance on the logarithmic transformation of the measurement (if the transformation successfully eliminated variance heterogeneity). Otherwise, analogous nonparametric methods, implemented in the computer program (StatXact; Cytel) were used along with appropriate control of error rates when making pairwise comparisons of groups. All pairwise comparisons are reported with P values based on a two-sided test of treatment effect, although a one-sided interpretation is valid and is of primary interest.

RESULTS
Epithelial Cell Death Induced by the Slight Ocular Irritant

Macroscopic Examinations and In Vivo Confocal Microscopy. The normal cornea, when observed in standard sagittal histologic section (Fig. 1A), showed an anterior corneal epithelial cell layer, five to seven cells thick, composed of basal epithelial cells (one cell), wing cells (two to three cells), and superficial epithelial cells (two to three cells), which overlaid a much thicker stroma composed predominantly of orthogonally arranged collagen lamellae with interspersed keratocytes. The posterior surface of the cornea was lined by a single layer of endothelial cells. Three-dimensional display of CMTF images from the same eye taken before death showed a similar organizational pattern but with brightly reflecting, large, superficial corneal epithelial cells covering the anterior surface (Fig. 1B, arrows). Below the superficial epithelium, a dark region of low reflectivity corresponding to basal and wing cells was seen separating the superficial epithelium from the slightly more reflective, underlying basal lamina (curved arrow) and stroma. Posteriorly, the cornea was lined by brightly reflecting endo-

### Table 1. Comparison of Normal and Slight Ocular Irritation 3 Hours after Treatment

<table>
<thead>
<tr>
<th>Subject</th>
<th>ET (μm)</th>
<th>Epithelial cell size (μm²)</th>
<th>CT (μm)</th>
<th>L/D</th>
<th>Clinical Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>46.8 ± 1.3</td>
<td>1538.9 ± 188.4</td>
<td>323.3 ± 6.7</td>
<td>16 ± 14.3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>44.5 ± 3</td>
<td>1385 ± 86.8</td>
<td>355.4 ± 2.3</td>
<td>13.8 ± 10</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>43.3 ± 2.9</td>
<td>1328.9 ± 94.2</td>
<td>330.3 ± 5</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>39.7 ± 2.1</td>
<td>1456.1 ± 194.1</td>
<td>347.4 ± 14.3</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Average</td>
<td>43.6 ± 3</td>
<td>1427.2 ± 90.7</td>
<td>339.1 ± 14.3</td>
<td>14.9 ± 1.6</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Slight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>38.4 ± 2.7</td>
<td>500.0 ± 156</td>
<td>371.1 ± 12.4</td>
<td>71.5 ± 3.7</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>42.0 ± 2.1</td>
<td>645.2 ± 276.4</td>
<td>353.4 ± 4.1</td>
<td>50.5 ± 21.4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
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<td>461.9 ± 94.1</td>
<td>387.1 ± 18.3</td>
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</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>5</td>
<td>42.8 ± 1.9</td>
<td>1023.2 ± 162.3</td>
<td>380.3 ± 12.4</td>
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<td>0</td>
</tr>
<tr>
<td>6</td>
<td>40.1 ± 2.7</td>
<td>592.8 ± 135.4</td>
<td>390.9 ± 8.1</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Average</td>
<td>41.2 ± 2.6</td>
<td>630.0 ± 203.4†</td>
<td>374.5 ± 12.3*</td>
<td>59.8 ± 13.2*</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

*P < 0.05.
†P < 0.001.
ET, epithelial thickness; CT, corneal thickness; L-D, live-dead assay; ND, not determined.
FIGURE 2. In vivo micrographs of surface epithelium (A, D) and laser micrographs of calcein-AM (B, E)-stained and ethidium homodimer (C, F)-stained corneas from control (A, B, C) and slightly irritated (D, E, F) corneas 3 hours after application. Laser micrographs show maximum intensity volume renderings (x-y) and representative sagittal sections (12 μm thick) from which cells were counted. Scale bar, 100 μm.

As previously shown, topical application of the slightly irritating surfactant resulted in loss of surface corneal epithelial cells, as is shown in the conventional histologic section by a reduction in the number of cells comprising the corneal epithelial layer (Fig. 1C). Erosion of surface epithelial cells did not, however, cause a marked decrease in epithelial layer thickness, measured by in vivo confocal microscope, because of apparent swelling or hydropic degeneration of newly exposed wing and basal cells. A corresponding decrease in epithelial thickness has been seen in other in vivo CM studies with this material.\(^{15,16}\) By 3-D display of CMTF images, loss of surface cells exposed the smaller, underlying wing cells indicated by the increase in the number of cells covering the epithelial surface (Fig. 1D, arrows). Quantitative analysis revealed a reduction in epithelial thickness to 41.2 ± 2.6 μm (not statistically significant), and a statistically significant decrease (\(P < 0.001\)) in the average surface epithelial cell size to 630 ± 203.4 μm\(^2\) (Table 1). Beneath the epithelial layer, there was no apparent change in the stroma or endothelium, although a slight but significant increase in average corneal thickness was noted (374.5 ± 12.3 μm; \(P < 0.01\)). The relative absence of stromal changes de-

In the normal rabbits, the epithelial and corneal thicknesses averaged 43.6 ± 3 μm and 339.1 ± 14.3 μm, respectively, whereas surface epithelial cell size averaged 1427.2 ± 90.7 μm\(^2\).

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ected by histologic study or by in vivo CM was consistent with the macroscopic observations in which all corneas were unremarkable (corneal score, 0); however, abnormal conjunctival changes were detected, evidenced by increased total scores of $6 \pm 1.4$.

**Ex Vivo Live–Dead Assays.** In the L-D assay, normally large surface corneal epithelial cells similar to those seen before death by in vivo CM (Fig. 2A), took up the cell-permeable calcein-AM and enzymatically converted the nonfluorescent probe to the intensely fluorescent polyanionic calcein, which produced a uniform green (approximately 530 nm) fluorescence throughout the cell (Fig. 2B). It was noted in the control corneas, however, that calcein-AM did not appear to penetrate below the intact surface epithelium, perhaps because of the presence of tight junctions between surface epithelial cells. Ethidium homodimer, on the other hand, entered only the cells with damaged membranes; binding to nucleic acids and showing a 40-fold increased in red fluorescence (approximately 600 nm). In the control corneas, occasional nuclei showed staining with ethidium homodimer, averaging $20.9 \pm 3.2\%$ of the total surface epithelial cells in the eight regions from two eyes that were evaluated (Fig. 2C). This number is higher than that previously reported for normal, undamaged corneas stained in situ. The difference between our findings and those in previous studies may have been caused by irritation of the ocular surface produced by in vivo confocal scanning. In addition, tissue blocks were stained ex vivo rather than in situ, which may have caused slight injury after removal and incubation.

Three hours after application of the slight irritant, exposed wing and basal epithelial cells, which appeared much smaller than the surface cells when visualized by in vivo CM (Fig. 2D), showed uptake of calcine-AM indicating the presence of viable epithelial cells (Fig. 2F). However, the number of ethidium homodimer-stained nuclei was significantly increased ($P < 0.01$). They averaged $49.2 \pm 4.5\%$ of the total surface epithelial cells, which suggests dramatic injury and cell death to the epithelial surface at 3 hours (Fig. 2F; Table 1). As expected, there was considerable variation in cell death among regions within the same eye, nevertheless there was a significant inverse correlation ($r = -0.78; P < 0.00001$) between the average size of the remaining, viable surface epithelial cells within a region and the percentage of cells staining with ethidium homodimer in the same region. Therefore, corneal surfaces covered by larger epithelial cells showed less cell death than did surfaces covered by smaller epithelial cells (Fig. 3A). A higher correlation ($r = -0.96; P < 0.005$) between percentage of cell death and epithelial cell size at 3 hours was obtained when the average value of the four sampled regions (central, inferior, nasal, and temporal) from individual eyes was used as an estimate of the area of involvement (Fig. 3B). This finding supports the importance of measuring area of involvement and depth of involvement. Although injury to the surface epithelium probably causes early desquamation of cells, many of the dead epithelial cells remained on the surface, indicated in the $y-z$ slice showing ethidium homodimer-stained nuclei overlying intact, calcine-AM-stained epithelium (Fig. 2F, y, z). Overall, the inverse correlations between cell size and ethidium staining seen at 3 hours supports the premise that early changes associated with slight surfactant-induced ocular irritation was caused by surface cell death, which exposed the smaller wing or basal epithelial cells.

Unlike observations at 3 hours, analysis of L-D stained corneas obtained from eyes 1 day after injury showed fewer ethidium homodimer-stained cells and no significant correlation between the percentage of cell staining and epithelial injury measured by in vivo CM (data not shown). There may have been no correlation because of the loss of damaged cells 24 hours after injury. In addition, repair of the damaged epithelial surface may have occurred through epithelial cell migration, cell proliferation, or both. Nevertheless, the data suggest that epithelial damage should be assessed within hours after exposure.

**Keratocyte Cell Death Induced by the Mild, Moderate, and Severe Ocular Irritants**

**Macroscopic Examinations and In Vivo Confocal Microscopy.** Macroscopic examination of treated eyes resulted in corneal and total scores of $21.7 \pm 13.3$ and $35 \pm 14.7$ for the mild irritant, $34 \pm 8.9$ and $47.8 \pm 8.8$ for the moderate irritant, and $40 \pm 0$ and $58.3 \pm 1$ for the severe irritant. These changes were significantly greater ($P < 0.05$) than scores from untreated control eyes (Table 2; control data not shown). Although there was a tendency for the cornea and eye to generate increased scores with increasing irritation potential at day 1, these differences were only significant for comparisons.
between mild and moderate and mild and severe irritants ($P < 0.05$ for both). There was no significant difference between eyes treated with moderate and severe irritants. This is consistent with previous historical data that indicate that eyes treated with moderate and severe irritants may achieve comparable maximum scores at day 1 but that there may be a difference in the length of time required for the cornea to become clear.\cite{16,17}

Histologic examination showed that increased severity in irritant was associated with an almost complete denudation of the corneal epithelium (Fig. 4). In eyes treated with the mildly irritating surfactant, some regions continued to show presence of intact epithelium, although it was markedly attenuated (Fig. 4A, arrow). As expected, in regions where the corneal epithelium remained intact, the underlying stroma appeared thinner compared with regions denuded of surface epithelium. For the most part, CMTF measurements were taken in regions devoid of epithelium (Fig. 4B) and showed significantly increased ($P < 0.05$) corneal thickness compared with that of control eyes, averaging 552.4 ± 77.6 \(\mu\)m (Table 2). Of note, 3-D display of CMTF scans showed increased reflections from the anterior keratocytes immediately underlying the basal lamina (Fig. 4B, arrowhead). These reflections, interpreted as degenerating and necrotic keratocytes, correlated with the presence of pyknotic nuclei in the anterior stroma, which were detected during histologic examination (Fig. 5A, arrow).

The moderately irritating surfactant showed fewer regions of intact epithelium; however, basal epithelial cells were evident in some regions (Fig. 4C, arrow), which, on higher magnification, appeared necrotic (data not shown). Measurements by CMTF revealed the mean corneal thickness to be significantly increased compared with that in control eyes ($P < 0.05$) averaging 588.0 ± 65.4 \(\mu\)m, which was greater than that of the mild irritant although not significantly different. Hyper-reflection or necrosis of anterior keratocytes seen by CMTF and histopathologic analysis was also noted, appearing slightly deeper than that detected after application of the mild irritant (Figs. 4D, 5B, arrows). By comparison, the severely irritating surfactant showed almost complete loss of keratocytes throughout the stroma in most specimens (Fig. 4E). Analysis by CTMF showed that corneal thickness was dramatically increased, averaging 796.8 ± 126.3 \(\mu\)m, which was significantly greater than that observed in control eyes and in mildly and moderately irritated eyes ($P < 0.05$). In addition, hyper-reflection of keratocytes was observed throughout the cornea, indicating extensive stromal injury (Fig. 4F).

Damage to keratocytes was further confirmed in single images from the CMTF scans, which showed that hyper-reflection of keratocytes seen in the 3-D display was related to the loss of normal keratocytes and the formation of punctate deposits consistent with degeneration and necrosis of keratocytes (Fig. 6). This change occurred in the anterior stroma of mildly (not shown) and moderately (Figs. 6A, 6B, 6C) irritated eyes, whereas in severely irritated eyes, keratocyte injury was detected deep within the stroma (Figs. 6D, 6E) extending to the corneal endothelium in some eyes. Using the transition zone between fragmented ($z = 11$, moderate; $z = 245$, severe) and normal appearing keratocytes as a measure of the depth of keratocyte injury, significant differences between mildly, moderately, and severely irritated eyes were observed (Table 2). In mildly irritated eyes, depth of injury averaged

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**Table 2. Comparison of Mild, Moderate, and Severe Ocular Irritation 1 Day after Treatment**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Cornea</th>
<th>Total</th>
<th>CT ((\mu)m)</th>
<th>Depth of Stromal Injury ((\mu)m)</th>
<th>L-D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>1</td>
<td>21</td>
<td>572.8 ± 47.7</td>
<td>35.3 ± 14.6</td>
<td>12.3 ± 21.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10</td>
<td>558.7 ± 20.4</td>
<td>27.5 ± 16.8</td>
<td>11.8 ± 13.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>55</td>
<td>522.0 ± 130.3</td>
<td>25.3 ± 18.2</td>
<td>13.8 ± 22.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>55</td>
<td>428.1 ± 151.7</td>
<td>17.8 ± 13.0</td>
<td>1.3 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>43</td>
<td>666.8 ± 6.2</td>
<td>49.3 ± 7.9</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>45</td>
<td>565.8 ± 170.6</td>
<td>29.8 ± 17.8</td>
<td>ND</td>
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<tr>
<td>Average</td>
<td>21.7 ± 13.3†</td>
<td>35.0 ± 14.7†</td>
<td>552.4 ± 77.6†</td>
<td>30.8 ± 10.7</td>
<td>9.8 ± 5.7</td>
</tr>
<tr>
<td>Moderate†</td>
<td>1</td>
<td>20</td>
<td>693.1 ± 27.6</td>
<td>47.8 ± 26.4</td>
<td>60.5 ± 47.2</td>
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<tr>
<td></td>
<td>2</td>
<td>51</td>
<td>522.4 ± 89.7</td>
<td>35.5 ± 28.6</td>
<td>20.0 ± 23.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>55</td>
<td>560.1 ± 126.2</td>
<td>59.0 ± 8.7</td>
<td>38.0 ± 24.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>47</td>
<td>603.6 ± 14.7</td>
<td>38.0 ± 14.7</td>
<td>27.3 ± 14.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>53</td>
<td>560.7 ± 28.1</td>
<td>55.8 ± 10.5</td>
<td>ND</td>
</tr>
<tr>
<td>Average</td>
<td>34.0 ± 8.9‡</td>
<td>47.8 ± 8.8‡</td>
<td>588.0 ± 65.4‡</td>
<td>47.2 ± 10.4</td>
<td>36.4 ± 17.7†</td>
</tr>
<tr>
<td>Severe</td>
<td>1</td>
<td>59</td>
<td>606.2 ± 79.5</td>
<td>573.4 ± 110.2</td>
<td>2.5 ± 4.4</td>
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<tr>
<td></td>
<td>2</td>
<td>57</td>
<td>886.7 ± 195.7</td>
<td>886.7 ± 195.7</td>
<td>5.0 ± 3.8</td>
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<tr>
<td></td>
<td>3</td>
<td>57</td>
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<td>755.4 ± 21.2</td>
<td>4.3 ± 4.3</td>
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<tr>
<td></td>
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<td>968.3 ± 44.7</td>
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<tr>
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<td></td>
<td>6</td>
<td>59</td>
<td>817.2 ± 122.1</td>
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<td>ND</td>
</tr>
<tr>
<td>Average</td>
<td>40.0 ± 0.01§</td>
<td>58.3 ± 11§</td>
<td>796.8 ± 126.3§</td>
<td>764.6 ± 156.9</td>
<td>14.1 ± 21.6</td>
</tr>
</tbody>
</table>

* Dead keratocyte cell count for mild and moderate; dead endothelial cell count for severe.
† Greater than control ($P < 0.05$).
‡ One rabbit died after 3-hour examination.
§ Greater than mild ($P < 0.05$).
|| Greater than mild and moderate ($P < 0.05$).
CT, corneal thickness; L-D, live-dead assay; ND, not determined.
FIGURE 4. Light micrographs (A, C, E) and confocal microscopic thru-focusing three-dimensional displays (B, D, F) of corneas 1 day after the application of mild (A, B), moderate (C, D), and severe (E, F) ocular irritants. Attenuated but intact epithelium was noted in some regions treated with the mild irritant (A, arrow), whereas treatment with the moderate and severe irritants caused necrosis of the basal epithelial cells (C, arrow) or complete denudation (E). Marked loss of keratocytes to a depth greater than 80% was also detected after the application of the severe irritant (E). Note the presence of hyper-reflective keratocytes in the anterior corneal stroma in the three-dimensional displays of corneas treated with mild and moderate irritants (B, D, arrows), whereas keratocytes appeared hyper-reflective throughout the cornea after the application of the severely irritating surfactant. Scale bar, 100 μm.

Ex Vivo Live-Dead Assays

L-D staining of corneal tissue from mildly irritated eyes (Fig. 7) showed the presence of live (calcein-AM-stained) and dead (ethidium homodimer-stained) cells. In some regions, intact surface epithelium was observed that contained viable epithelium and occasional dead epithelial cells. Of interest, dead cells appeared to align along the leading edge of the epithelial sheet (Fig. 7, dead, arrow). Within the exposed stroma and depending on the region evaluated, varying numbers of keratocyte nuclei were stained with ethidium homodimer (dead, arrowheads). In x-z slices from the volume renderings, the number of dead keratocytes could be counted (dead, arrows). In the mildly irritated group, the number of dead keratocytes identified in the four regions of each eye averaged 9.8 ± 5.7 cells (Table 2). In those regions showing only a few dead cells, it is interesting that underlying viable keratocytes appeared to have a higher cell density.

In moderately irritated eyes, depth of injury was significantly greater than that observed in mildly irritated eyes, averaging 47.2 ± 10.4 μm (P < 0.05). The severely irritated eyes showed very deep keratocyte injury to an average depth of 764.6 ± 156.9 μm at day 1, which was significantly greater than that seen in mildly or moderately irritated corneas (P < 0.05). These differences are consistent with previous findings. Normalizing depth of injury for differences in corneal swelling did not change the level of significance.
compared with regions with greater cell death. Furthermore, in the x-z slice, viable keratocytes appeared to have a higher density at the surface (live, arrows) and a lower density below the anterior stroma. This finding is consistent with earlier reports that keratocyte density is highest at the anterior stroma and decreases markedly posteriorly.27 L-D staining of specimens from moderately irritated eyes generally showed more dead keratocytes in most regions than were identified in mildly irritated eyes 1 day after treatment (Fig. 7, dead). Overall, in the four eyes evaluated by L-D, there was significantly greater (P < 0.05, mild versus moderate) keratocyte death, averaging 36.4 ± 17.7 cells/region (Table 2). In x-z slices, dead keratocytes also extended to a greater depth than that seen with mild irritation, which confirms the observation made during in vivo CM that the depth of injury with the moderate irritant was greater than that observed with the mild irritant. Viable keratocytes appeared similarly at a lower density and deeper within the stroma than that seen after mild injury (Fig. 7, Live), again consistent with the observation that density of keratocytes is less in the deeper portions of the cornea.

Application of the severe irritant resulted in marked death of keratocytes to a much greater depth than that evaluated (170 μm, z) in this study (Fig. 7, dead). Within this region, no viable keratocytes were identified (Fig. 7, live). These findings were consistent with the in vivo confocal microscopic measurements revealing the depth of corneal injury to be 764.6 μm, far beyond the 170 μm depth measured in the L-D assay (Table 2). Overall, these observations confirmed that the depth of injury was greater than 170 μm, and that the fragmentation of keratocytes observed by in vivo confocal microscope was principally caused by cell death and not infiltration of inflammatory cells or changes in the extracellular matrix. Because it was impossible to quantify the number of dead keratocytes using laser scanning CM (all were more than 100 cells/region), damage to the corneal endothelium was determined by imaging the endothelial rather than the epithelial side of the corneal button. The application of the severe irritant caused a range of cell death varying from a few cells to almost the complete endothelium (Fig. 8), averaging 14.1 ± 21.6 cells/region. It is interesting to note that the cornea with the greatest endothelial cell death also showed the greatest corneal swelling (668.3 μm) and a 100% depth of injury, measured by in vivo CM.

Depth of injury measured by in vivo CM and the number of ethidium homodimer-stained nuclei counted in each sample (Fig. 9) had a significant correlation coefficient of 0.79 (P < 0.00001). Furthermore, normalizing depth of injury for differences in corneal thickness did not change the correlation. Overall these observations confirmed that the area and depth of injury measured by in vivo CM is an accurate measure of the extent of cell death caused by application of irritating surfactants.

**DISCUSSION**

Based on results in earlier studies, we have proposed that ocular irritation is dependent on the initial area and depth of injury produced by surfactants of differing potential to cause irritation.15,16,18 Specifically, studies using in vivo CM have shown that there are significant differences in the depth of initial corneal injury among ocular irritants: Slight irritants damage the corneal epithelium; mild irritants damage the corneal epithelium and superficial stroma; moderate irritants damage the corneal epithelium and anterior stroma; and severe irritants damage the corneal epithelium, stroma, and endothelium.15,16 In these studies, in vivo assessment of injury was based on the disappearance of corneal epithelium and stromal keratocytes and the appearance of punctate stromal deposits presumed to be degeneration and necrosis of keratocytes.

In the present study, we have confirmed and extended our earlier observations to demonstrate that the initial corneal injury produced by irritating surfactants at 3 hours and 1 day after exposure is directly correlated to the extent of cell death measured by the L-D assay. Furthermore, surfactants that cause differing degrees of irritation produce significantly different amounts of corneal cell death. Slightly irritating surfactants cause less cell death than mildly irritating substances, and so on, with severely irritating substances causing the most cell death. Overall, these findings suggest that surfactant-induced ocular irritation can be defined quantitatively by the area and...
FIGURE 6. Two-dimensional in vivo micrographs from a moderately (A, B, C) and a severely (D, E, F) irritated cornea taken at different depths on day 1 after treatment. In the moderately and the severely irritated corneas, there is loss of surface epithelium (A, z = 0 μm; D, z = 0 μm, respectively) and there are punctate deposits in the anterior stroma (B, z = 11 μm; E, z = 243 μm), consistent with keratocyte degeneration and necrosis. Note that the reappearance of normal keratocytes occurs more anteriorly in the moderately irritated cornea (C, z = 91) than in the severely irritated cornea (F, z = 443), suggesting a deeper injury caused by the severe irritant. Scale bar, 100 μm.

Depth of cell death. Moreover, the associations between extent of initial injury and subsequent responses as shown recently by Maurer et al.18 in groups of rats treated with these surfactants suggest that the extent of initial cell death plays a critical mechanistic role in determining the ocular irritation response to surfactants. Therefore, assessment of the area and depth of corneal cell death may provide an important basis for the development and validation of future mechanistically based in vitro tests to replace the use of animals for ocular irritation testing. Future work extending these findings to show that area
FIGURE 7. Laser micrographs of dead and live cells in mildly, moderately, and severely irritated corneas 1 day after application. In general, there were fewer dead keratocytes (arrowheads) seen with the maximum intensity projection (xy) or sagittal section (xz) after the application of the mild compared with the moderate irritant. In addition, there was a higher density of live keratocytes (arrows) in the mildly irritated corneas, which is consistent with a higher density of keratocytes in the anterior stroma. The application of the severe irritant resulted in complete loss of live keratocytes in the volume evaluated. Scale bar, 100 μm.

and depth of injury predict subsequent response in the same eye and that a similar correlation exists with a diverse array of surfactants of differing irritation potential and type (cationic, anionic, and nonionic) will be important in clearly determining the broad applicability of this hypothesis to surfactant irritancy testing. In addition, evaluation of a wide range of irritating materials of differing chemical basis including acids, alcohols, and alkalis, will be important in further determining that cell death is a general mechanistic basis for the ocular irritation response.

To our knowledge, this is the first study in which the L-D assay has been used to assess quantitatively ocular irritation potential. Previous studies in the eye have used calcin-AM and ethidium homodimer staining of cells to identify the in situ organization of keratocytes and the apoptosis of normal superficial corneal epithelial cells and Fas/Fas ligand–stimulated cultured keratocytes. Recently, Ren and Wilson have shown that approximately 1% of the normal superficial corneal epithelial cell population stains with ethidium homodimer, which indicates a slow rate of epithelial apoptosis before cell shedding. A similar staining pattern was confirmed with the TdT-dUTP terminal nick-end labeling (TUNEL) assay, which identifies DNA fragmentation characteristic of apoptosis. In the present study, a higher level of staining with ethidium ho-
modimer was identified in control corneas. This higher level could be caused by manipulation of the eye during in vivo microscopy and ex vivo staining. Although care was taken in the contact of the objective to the corneal surface and earlier studies have not shown a significant effect of CM on the ocular surface, decreased surface epithelial cell size has been noted after repeated examination by confocal microscope. In addition, tissues were removed before L-D staining in contrast to staining in situ as reported by Ren and Wilson. This may have lead to retention and better surface labeling of cells or increased damage to surface epithelial cells and may have resulted in a higher number of ethidium homodimer-stained epithelial cells. Nevertheless, small potential artifacts induced by manipulation should be relatively equal across groups and would not account for the large differences between control eyes and slightly irritated eyes that was observed in this study. Furthermore, these factors should not directly affect measurement of keratocyte death, because these cells are below the surface of the cornea and are protected from any accidental contact.
This study further validates the use of in vivo CM to quantify area and depth of corneal injury objectively. In vivo CM of the cornea has been used previously to assess quantitatively the effects of contact lens wear by measuring epithelial cells size, to assess corneal endothelial wound healing by measuring wound diameter, and to determine the mechanism of refractive change after radial keratotomy by measuring wound gape. More recently, in vivo CMTF has been used to quantitate corneal epithelial regeneration, stromal repair, and corneal haze after excimer laser photorefractive surgery. In vivo CM has also been used in clinical diagnosis of ocular infection with Acanthamoeba and Aspergillus and in identifying corneal epithelial nerve patterns. In all these studies, in vivo CM has provided valuable and at times otherwise unattainable quantitative microscopic information that can be directly correlated to clinical observations. The continued development and application of this new microscopic technique will certainly improve our ability to diagnose, to follow up treatment, and to understand the underlying cellular mechanism of corneal disease and function.

Overall, our findings highlight and directly affect two important aspects in the development and validation of mechanistically based in vitro alternatives, thus emphasizing the need for a better understanding of the cellular and molecular mechanisms of in vivo ocular irritation tests. First, in vitro assays have not been developed on the basis of known mechanisms of in vivo ocular irritation. In some instances, such assays have been developed without regard to possible mechanisms or biologic processes specifically involved in ocular irritation. In the present study, significant differences among surfactants with differing severity of irritant were detected on the basis of area and depth of cellular injury specifically associated with extent of cell death. If cytotoxicity is generally applicable to surfactants and is broadly applicable to ocular irritants, then in vitro alternative tests that are not predicated on cytotoxicity may not be helpful. Furthermore, tests now predicated on cytotoxicity may need modification to determine specifically the area and depth of injury.

Second, validation of in vitro assays using data from animal ocular irritation tests has been problematic. Validation of in vitro tests has been by correlative analysis to the maximum average score, which is the highest mean score during an in vivo ocular irritation test, regardless of time. The maximum average score is then a single, weighted number, used to order by rank the irritation potential of a variety of test materials, even though the score may be achieved at different times in different materials, and it does not mechanistically discriminate among a variety of primary and secondary responses for the cornea, iris, and conjunctiva. Our findings suggest that the more appropriate criterion for validating in vitro alternatives may be area and depth of initial injury and more specifically, cytotoxicity within the cornea.

In conclusion, the present study establishes for the first time a direct correlation between the initial in vivo effects of ocular irritation and the ability of the same irritant to cause cell death within the cornea. This finding strongly suggests that corneal cell death is an important if not the primary mechanistic determinant of initial surfactant-induced ocular irritation. Based on these findings, we propose that the development of in vitro models that are based on the measurement of area and depth of cell injury may provide a mechanistic basis for developing and validating in vitro assays to replace the use of animals for surfactant-induced ocular irritation and, perhaps more broadly, for a testing diverse array of irritants. Our current research plans include establishing the correlation between extent of cell death and subsequent response in the same eye and determining whether the correlation between cell death and ocular irritation is true for other surfactants of differing type (cationic, nonionic, and ionic) and for different types of irritants (acid, alkali, and alcohol).

References