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THE USE OF CELL LYSIS AS AN INDEX OF OCULAR IRRITATION POTENTIAL

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Abstract

We report an *in vitro* method for assessing ocular irritation by measuring cell lysis of mouse connective tissue (strain L, Clone 929) and rabbit corneal cell lines (SIRC). Lysis of corneal epithelial cells *in vivo* leads to swelling and subsequent opacification of the underlying corneal stroma. *In vitro* lysis was determined by the measurement of changes in absorbance (360 nm) of cell suspensions over a 10-min exposure to test materials. These data were expressed as numbers of cells/ml using linear regression. The regression line obtained was linear and similar for both cell types. Loss of viability and spontaneous cell lysis were minimal over the assay interval. In a separate study, rabbit corneal cells were used to assess alteration of cell membrane integrity following 30-min exposures to test materials by measuring trypan blue dye exclusion in these cells. Activities of test materials were ranked according to the lowest concentration capable of producing statistically significant cell lysis as follows: triethanolamine lauryl sulfate (TLS) < triethanolamine (TEA) < propylene glycol (PG) (0.003, 0.1, and 10%, respectively, $p < 0.01$). The order was similar when dye

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exclusion ability was the index of activity except that benzalkonium chloride (BAK) was more active than the other materials. BAK and materials with cationic properties were not easily tested in the proposed suspension assay because of increased absorbance due to protein denaturation and subsequent masking of dissolution, if present. Six commercial formulations coded A through F were tested in the suspension assay as 1:3,000, 1:1,000, and 1:300 dilutions and compared to similarly diluted standards to assess cell dissolution ability. A shampoo concentrate acted similarly to TLS and an amphoteric-surfactant-based shampoo acted similarly to TEA at dilutions of 1:3,000. Effects of other formulations were intermediate between these extremes ($p < 0.05$), but similar to one another. At 1:1,000 dilutions, the effects converged, with all formulations having the same activity, which was significantly less than that of TLS, but significantly greater than that of TEA ($p < 0.05$). Over the next interval, the effects paralleled that of TLS but were significantly less except for that of the concentrate ($p < 0.05$). Another formulation showed a plateau-like effect while the remaining one showed a decreased effect owing to its inherent cationic nature. Results were in general agreement with *in vivo* and *in vitro* observations as well as with known chemical activities of the materials. A method for estimating the relative ability of materials and formulations to cause cell lysis is proposed which may serve as a model for the assessment of a form of corneal opacity initiated by the loss of integrity of limiting corneal layers.

Introduction

As noted in previous reports,[1,2] there is great interest in the development of *in vitro* alternatives to testing procedures requiring animals. We have recently reported two *in vitro* methods for the assessment of irritancy potential of drug and cosmetic materials. One of these methods assessed the change in light absorbance at 360 nm by rabbit corneal cell monolayers (SIRC cell lines), which was considered to be an index of corneal opacity produced by the precipitation of cellular components.[1] Since initial monolayer absorbances were low, the method was insensitive to the effects of materials (detergents, anionic or nonionic surfactants) causing dissolution of the monolayer (unpublished data). Either mechanism (precipitation or dissolution) can cause corneal opacity,[3,4] which is a significant factor in ocular irritancy.[5] In the latter case, the loss of limiting corneal layers has been shown to create opacity as a result of swelling of the underlying stromal layers.[4]

A number of cytotoxicity tests have reported the use of cell suspensions.[6–10] With their use, the initial absorbance could be increased as required. Mouse connective tissue cells (strain L, clone 929) are known to grow readily in suspension and were used as suspensions in a cytotoxicity assay.[9] Other reports[11,12] have demonstrated the linear nature of the relationship between cell concentration and absorbance, one which satisfied Beer's law in low cell concentrations, that is, OD < 0.05.

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Cell suspension techniques have been used previously to assess cytotoxicity. They have been used to assess antimicrobial and/or surfactant hemolytic potential.[6, 10, 13] Suspensions have been used to assess ocular irritancy potential by measuring release of polymorphonuclear leukocyte granules into solution[8] and by determining surfactant concentrations causing a loss of viability of 50% of suspended strain L, clone 929 cells.[9]

This paper reports the development of a method for assessing ocular irritation potential *in vitro* using strain L, clone 929 cells and its subsequent modification to allow use of SIRC rabbit corneal cells. The test materials used were chosen on the basis of their known chemical activity and irritancy potential both *in vitro* and *in vivo*. They included benzalkonium chloride (BAK), triethanolamine lauryl sulfate (TLS), triethanolamine (TEA), and propylene glycol (PG). The ability of this method to be used in the testing of commercial formulations was assessed by exposing cell suspensions of rabbit corneal cells to fixed dilutions of commercial shampoos (1:3,000, 1:1,000, 1:300). Similarly diluted standards of high and low irritancy potential were included for relative comparisons.

Finally, we also report results of parallel investigations of cytotoxicity which assessed the ability of rabbit corneal cell suspensions to exclude trypan blue dye following exposure to the above standards. This particular method was used to assess cell membrane integrity, the disruption of which may be an early event in cytotoxicity.[14] Presumably, changes in dye exclusion ability would precede or at least parallel the response of cell lysis.

The method reported herein may be useful in screening compounds for their ability to cause ocular irritation by their solubilizing effect on corneal cells. The present method was relatively easy to perform, was able to differentiate among the chosen test materials, and allowed for the testing of commercial formulations. It complements our methodology to assess irritation produced by materials causing cellular precipitation. The ability of rabbit corneal cells to exclude trypan blue dye following exposure to irritants both confirmed our observations and underscored the importance of cell membrane integrity as an index of irritancy potential.

Materials and Methods

Cells

Two certified cell lines were used: SIRC rabbit corneal cells (ATCC CCL60) and strain L, clone 929 mouse connective tissue cells, clonal L-cell line (ATCC CCL1). They were purchased from the American Type Culture Collection (Rockville, MD). These cultures were grown as adherent monolayers in a forced-air incubator maintained at 37 °C. Growth medium was that specified by the supplier with the addition of 0.05 mg/ml gentamicin or 10,00 U/ml penicillin and 10,000 μg/ml streptomycin

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to SIRC and strain L, clone 929 media, respectively. All manipulations were performed using sterile technique. Stock cultures were maintained in 80 cm^2 sterile polystyrene T-flasks (Nunclon) and were fed once per week with growth medium. At this time flasks of SIRC and strain L, clone 929 cells were divided into two or three equal parts, respectively, and recultured. A mixture of 8% CO_2 in air was introduced into each flask containing mouse connective tissue cells before the flasks were sealed. All sera used were inactivated at 56°C for 30 min.

Cell Lysis Determinations

Suspension Medium and Test Material Preparation

The suspension medium used throughout this assay consisted of Dulbecco's phosphate-buffered saline solution without calcium (calcium-free PBS), adjusted to pH 7.3, supplemented with 5% fetal bovine serum (K. C. Biologicals), and filtered through a 0.22-μm filter (Millipore Corp.). Concentrations of test materials in the calcium-free PBS medium were five times that desired in the assay tubes to allow for subsequent procedural dilution during the initiation of the assay. Materials tested included TLS, TEA, PG, and BAK in concentrations ranging from 0.001 to 10%. Shampoo formulations were those commercially available, diluted in calcium-free PBS-Ca so that cells were exposed to 1:300, 1:1,000, and 1:3.000 dilutions. All other materials were of standard reagent or manufacturing grade.

Preparation of Mouse Cell (Strain L, Clone 929) Suspensions

Cells were used after 6 days of growth as above. As a rule, one flask of cells was used for each concentration level tested. Flasks were treated with 0.125% trypsin-EDTA and the cells obtained were suspended to 20 ml with calcium-free PBS medium, pooled, and maintained at 37°C until use.

Preparation of Rabbit Corneal Cell (SIRC) Suspensions

Cells were used after 5 days of growth. Suspensions were prepared as above except that two flasks of cells were used for each concentration level tested, each flask was washed three times with 0.1% EDTA in phosphate-buffered saline, pH 7.4, before the addition of 0.125% trypsin-EDTA, and the cells obtained were resuspended to 10 ml with calcium-free PBS medium.

Preliminary Experiments

Viability was determined by trypan blue dye exclusion by cells before and after exposure to assay conditions. Results were analyzed by a one-way analysis of variance.

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