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Comparison of an Immortalized Human Corneal Epithelial Cell Line and Rabbit Corneal Epithelial Cell Culture in Cytotoxicity Testing

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ABSTRACT

The cytotoxicity of benzalkonium chloride (BAC) and disodium edetate (EDTA) was evaluated *in vitro* in rabbit corneal epithelial primary cells and in the immortalized human corneal epithelial cell line SV40. Cell injury was assessed by lactate dehydrogenase (LDH) leakage and by reduction of the tetrazolium salt WST-1 to formazan by mitochondrial metabolic activity. Cell cultures were exposed to test compounds both in serum-free and in serum-containing medium. Although WST-1 and LDH tests measured different physiological endpoints, they yielded comparable results. However, the LDH test seemed less reliable due to great variation. The use of serum was found to result in lower toxicity of the compounds in both tests. The rabbit primary cell culture and the human corneal cell line were quite similar in their responses to BAC and EDTA. The human cell line is a promising *in vitro* alternative in oculotoxicity testing.

INTRODUCTION

The Draize eye irritation test (1) is based on the scoring of the responses of rabbit eye cornea, conjunctiva and iris to test substances. The Draize test has been extensively criticized in the literature for its subjectivity, lack of discrimination of fine response differences, and overestimation of the human response (2–5). The reproducibility of the Draize test has also been found to be poor both within and among laboratories (6–9).

In recent years, ethical considerations have increased the need to reduce the use of laboratory animals in the safety testing of various chemicals and formulations. In search of alternatives for laboratory animals, many researchers have focused on cytotoxicity assays that are simple and reproducible and yield a clearly defined endpoint. Measurements of various physiological endpoints have been introduced (10).

As epithelial cells form the outermost layer of the cornea and, thus, are readily exposed to injury, they are a very promising alternative for *in vitro* cytotoxicity testing. Primary cultures of rabbit corneal epithelial cells have been extensively used for the assessment of potential ocular irritation (11–12). The use of human corneal epithelial cells has been restricted, partly due to the limited availability of human corneas. Furthermore, culturing human corneal epithelium has been found problematic with the same methods that work well with the rabbit (22). The disadvantage of primary cells is their short life span which increases the need for seeding new primary cultures.

In the present study, we used a human corneal epithelial (HCE) cell line SV40, and compared it to the rabbit corneal epithelial (RCE) cell culture. The choice of these culture models was made because rabbit is the generally used species in eye toxicology, and because there is a need to find a test using human cells and tissues as methods in the safety evaluation of drugs. Benzalkonium chloride (BAC) and disodium edetate (EDTA) were used as test compounds when evaluating the culture models and cytotoxicity tests. BAC and EDTA are commonly used in topical ophthalmic preparations. BAC is a cationic surfactant and a very effective antimicrobial agent that is widely used as a preservative in many eye drops. Like many other surfactants, BAC exerts its bactericidal effects by destroying bacterial cell walls. *In vivo* studies have shown that BAC is a severe eye irritant. EDTA is a known calcium chelator. To ophthalmic preparations, EDTA is added for stability purposes, and it has been used for removal of the superficial calcific opacities and as an aid for the decontamination of the eye (23). Cell injury caused by BAC and EDTA was assessed with lactate dehydrogenase (LDH) leakage as an index of cell membrane integrity and with reduction of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to formazan as an index of mitochondrial metabolic activity. The effects of different factors (exposure time, recovery time and serum content in the medium) on the test results were evaluated.

MATERIALS AND METHODS

Rabbit Corneal Epithelial Cell Culture

Primary cultures of rabbit corneal epithelial cells were established with an endothelium-free explant method. This method was modified from the methods described by Ebato *et al.* (22), Araki *et al.* (24), and Kahn *et al.* (25). Male New Zealand rabbits were sacrificed by intravenous sodium pentobarbital injection through the ear vein. The lamellar corneal button was excised and rinsed in Hank's balanced salt solution (HBSS) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) (from Gibco, Paisley, UK), then the endothelium side of the cornea was discarded. The epithelium was cut into twelve segments in a culture dish and let to dry for 5 minutes to insure a good attachment of the tissue segments to the dish. All the cell culture equipment (petri dishes, culture flasks, multiwell plates) were obtained from Nunc (Roskilde, Denmark). The culture medium contained 1 vol of Dulbecco's modified Eagle's medium (DMEM) and 1 vol of Ham's nutrient mixture F-12 (Gibco). The medium was supplemented with 15% (vol/vol) heat-inactivated fetal bovine serum (FBS, Gibco), 1% (vol/vol) antibiotic, antimycotic solution (penicillin 10,000 U/ml, streptomycin 10,000 μg/ml and amphotericin B 25 μg/ml, Gibco), 2 mM L-glutamine (Gibco), 5 μg/ml insulin (Sigma, St. Louis, MO), and 10 mg/ml human epithelial growth factor (EGF, Sigma). The medium was carefully added upon each tissue segment with a Pasteur pipette, and the tissue was allowed to incubate in 5% CO_2/95% air at 37°C in a humidified atmosphere. On the next two days, medium was carefully added on the tissue segments using a Pasteur pipette. Outgrowth was normally observed on the third day. When cells migrating away from the explants were observed, the tissue segments were aseptically removed with forceps in order to avoid fibroblast contamination, and 2 ml of medium was added. The whole of the medium was changed within 3 days and then every 2 days. About two weeks after the culture was initiated, the cells were transferred into 25 cm^2 T-flasks. Incubation was continued at 37°C in 5% CO_2/95% air. The medium was changed every 2nd day until

the cell culture reached confluence. Due to their limited life span, RCE cells were stored in liquid nitrogen for later use. After cryopreservation, the cell culture was first initiated in a 25 cm^2 T-flask and then plated at a density of 15,000–20,000 cells/well in a 96-well flat bottom microwell plate. After plating, the cells were allowed to grow for 24 hours. Cytotoxicity tests were conducted with passage three cells before the cells in culture had reached full confluence.

Human Corneal Epithelial Cell Line

An immortalized human corneal epithelial cell line (HCE) was established by Araki-Sasaki and colleagues (26) by infecting primary human corneal epithelial cells with a recombinant SV40-adenovirus vector and by cloning three times to obtain a continuously growing cell line. The HCE SV40 cell line used in this study was a generous gift of the research group of Araki-Sasaki and co-workers. Initially, HCE cells were grown in a culture medium containing 1 vol of Dulbecco's modified Eagle's medium and 1 vol of Ham's nutrient mixture F-12 supplemented with 15% (vol/vol) fetal bovine serum, 1% (vol/vol) antibiotic, antimycotic solution (penicillin 10,000 U/ml, streptomycin 10,000 μg/ml and amphotericin B 25 μg/ml), 2 mM L-glutamine, 5 μg/ml insulin, 10 ng/ml human epithelial growth factor, 0.5% (vol/vol) dimethylsulfoxide (DMSO, Sigma), and 0.1 μg/ml cholera toxin (Calbiochem, San Diego, CA). The cells were grown in 80 cm^2 T-flasks and serially passaged at a split ratio of 1:5 twice a week. For cytotoxicity assays, cells with passage numbers 49–81 were used. For cytotoxicity testing, HCE cells were grown in the same medium as RCE cells (without dimethylsulfoxide and cholera toxin) and plated at the density of 15,000–30,000 cells/well in a 96-well microtiter plate. The cells were exposed to the test compound-containing medium 24 hours after plating before the cell culture started to form multilayers and became confluent. DMSO and cholera toxin were eliminated from the growth medium to simulate better the natural physiological conditions. The elimination of DMSO and cholera toxin did not affect the morphology of the cell; however, a decrease in growth rate was observed.

Treatment Of Cell Cultures For Cytotoxicity Assays

BAC was manufactured by FeF Chemicals A/S (Køke, Denmark) and EDTA by Merck KGaA (Darmstadt, Germany). For cytotoxicity testing, the test compounds were dissolved in two kinds of mediums: serum-lacking medium or a medium containing 15% (vol/vol) fetal bovine serum. The original medium was discarded, and 100 μl of test compound-containing medium was added to the culture wells for a treatment period of five minutes or one hour. The test compound-containing medium was removed and used for lactate dehydrogenase leakage test. After the removal of the test compound-containing medium, the cells were at first rinsed once with basal medium (Dulbecco's modified Eagle's medium and Ham's F12, 1:1) without serum. In parts of the experiments, 100 μl of normal growth medium with 15% (vol/vol) FBS was added, and the cells were returned to the incubator for a one-hour recovery period, after which the WST-1 test was initiated. In other parts of the experiments, the WST-1 test was initiated immediately after medium change (no recovery).

LDH Test

LDH test was modified from the "Automated Analysis Boehringer Mannheim LD/LDH Assay" (Boehringer Mannheim GmbH Germany, cat. no. 191353). After the exposure period, 25 μl of test compound-containing sample was pipetted into a 96-well microtiter plate, and 250 μl of coenzyme/buffer containing 56 mM Tris buffer (pH 7.4), 0.17 mM NADH (disodium salt), and 5.6 mM EDTA (tetrasodium salt) was added. The reaction was started by the addition of 25 μl of 14 mM

pyruvate solution (monosodium salt). The average rate of disappearance of NADH (LDH activity) was monitored at room temperature by measuring the absorbance for 3 min at a wavelength of 340 nm with the Multiskan MS EIA reader (Labsystems Oy, Helsinki, Finland). Enzyme leakage into the medium was expressed as percentage of control.

WST-1 Test

The WST-1 test was based on a commercially available cell proliferation reagent WST-1 (Boehringer Mannheim, cat no. 1644807). The purpose of the WST-1 test was to determine the viability of the cells (cells left attached on the dish). Immediately after changing the medium or after a one-hour recovery period, 10 μl of WST-1 proliferation reagent was added on cells in normal medium. The cells were incubated in a humidified atmosphere at 37°C in 5% CO_2/95% air for two hours, after which the absorbances were measured using a wavelength of 450 nm with the Victor 1420 Multilabel Counter (Wallac Oy, Turku, Finland). The use of a two-hour incubation time was based on preliminary experiments conducted with the WST-1 reagent.

Statistics

Cytotoxicity data for the one-hour exposure were obtained from four to seven different experiments by testing six concentrations per plate with eight wells per concentration, while cytotoxicity data for the 5-min exposure were obtained from four different experiments by testing six concentrations per plate with sixteen wells per concentration. The mean optical density values corresponding to the non-treated controls were taken as 100%. The results of treated cultures were expressed as percentages of non-treated control cultures. EC_{50} values (%) were determined as the concentrations of test agents, which decreased the WST-1 reduction values to 50% of the control value (GraphPad Prism, GraphPad™, San Diego, USA). The statistical significance of the differences of the EC_{50} values between cultures exposed to test agents without FBS and with 15% FBS were determined with Student's two-tailed t-test (GraphPad Prism).

TABLE 1.
Toxicity of Benzalkonium Chloride (BAC) and EDTA in Corneal Epithelial Cells

Test agent	Cell type	Exposure time (min)	% FBS in exposure medium	Recovery time (min)	WST-1 EC_{50} values (%) (MEAN±SEM)		No of plates (N)
BAC	HCE	60	0	60	0.00108±0.00009	P=0.0025 **	7
		60	15	60	0.00197±0.00021		7
		5	0	60	0.00076±0.00011	P=0.0645	4
		5	0	0	0.00145±0.00035		2
	RCE	60	0	60	0.00106±0.00030	P=0.0178*	5
		60	15	60	0.00332±0.00066		6
EDTA	HCE	60	0	60	0.07855±0.00696	P=0.0304*	4
		60	15	60	0.09520±0.00252		6
	RCE	60	0	60	0.07886±0.01003	P=0.0738	5
		60	15	60	0.10870±0.00463		3

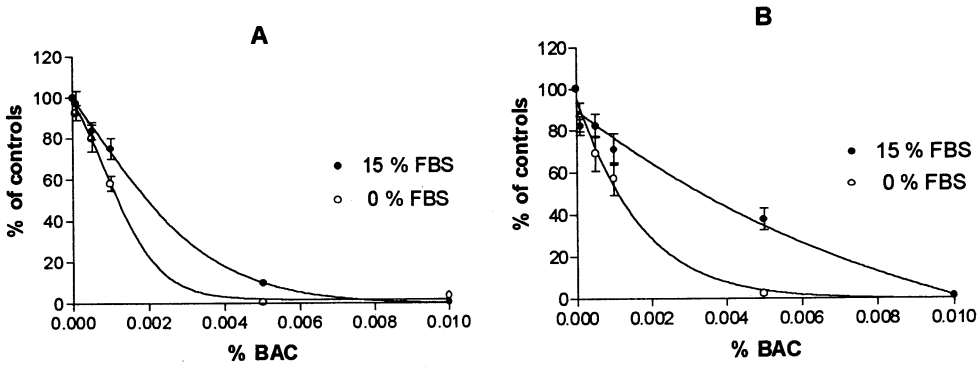

FIGURE 1. Reduction of WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) in Human Corneal Epithelial Cell Line (**A**) and Rabbit Corneal Epithelial Primary Cells (**B**) Exposed to Benzalkonium Chloride (BAC) for 1 hr. Recovery time after exposure was 1 hr.

RESULTS

WST-1 Formazan Formation

The test substances BAC and EDTA caused a concentration-dependent decrease in WST-1 formazan formation in both cell types. The EC values (%) determined are summarized in Table 1.

Exposure to 0.001% (w/v) BAC for one hour caused about 40% decrease of viability both in RCE cell culture and in HCE cells (Fig. 1). Both cell types were killed when treated with 0.005% (w/v) BAC for one hour. There were no differences between the EC_{50} values of the RCE cell culture and HCE cells exposed to BAC ($P > 0.05$). Serum [15% (v/v)] protected the cells from the toxic effects to some extent (Table 1).

EDTA was less cytotoxic to the corneal cell cultures than BAC (Fig. 2). A slight (10%) reduction in viability of RCE cell culture was detected when exposed to 0.05% EDTA. No such reduction was seen in HCE cells. EDTA (0.1%) reduced the viability of HCE cell cultures by about 90%, and RCE cells by about 80%. There were no statistical differences between the EC_{50} values of the EDTA treated human and rabbit corneal epithelial cell cultures ($P > 0.05$). Serum protected the cell cultures slightly from the EDTA toxicity (Table 1).

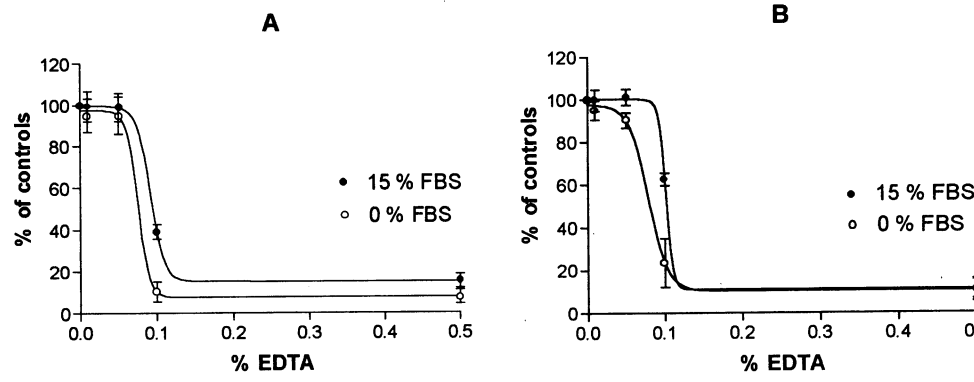

FIGURE 2. Reduction of WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) in Human Corneal Epithelial Cell Line (**A**) and Rabbit Corneal Epithelial Primary Cells (**B**) Exposed to EDTA for 1 hr. Recovery time after exposure was 1 hr.

Five min BAC treatment (Fig. 3A) caused an equally severe effect on HCE cells as one hour BAC treatment (Fig. 1A). In the WST-1 test performed immediately after five min BAC treatment, the cytotoxicity was less severe than in the test performed after one hour recovery time (Fig. 3A). However, the difference between EC_{50} values was not statistically significant ($P = 0.0645$, Table 1). Five min EDTA treatment (Fig. 3B) was far less cytotoxic to HCE cells than one hour treatment (Fig 2A). Moreover, the recovery period (1 hr) did not affect the cytotoxic effect of five min EDTA treatment.

LDH Leakage

Considerable LDH leakage was detected both in HCE cells (Fig. 4A) and RCE cell culture (Fig. 4B) starting from 0.005% BAC concentration. LDH was found to leak more in RCE cell culture than in the HCE cells subjected to BAC. Serum protected cells from LDH leakage. In serum-containing medium, no increased leakage of LDH was found when HCE cells were exposed for one hour to 0.005% (w/v) BAC or less. Five min exposure to BAC (0.01% w/v) (Fig. 6A) caused similar leakage of LDH as 1 hr exposure. Higher concentrations (0.05% w/v and 0.1% w/v) reduced the activity of LDH in the medium after five min exposure.

In EDTA-treated cells, LDH leakage increased when the cells were treated with 0.1% (w/v) EDTA or above for one hour (Fig. 5). LDH leaked more in RCE cell cultures than in HCE cells. As with BAC, great variations were found in LDH leakage when the cells were exposed to EDTA in serum-lacking medium. Serum protected HCE cells from LDH leakage in the whole concentration range of EDTA (0.01–1% w/v). In RCE cell cultures, LDH leakage increased, whether or not serum was added, starting from 0.1% (w/v) EDTA concentration. Five min exposure to EDTA caused only a small effect in the WST-test (Fig. 6B).

DISCUSSION

The evaluation of cell toxicity with *in vitro* methods is based on the measurements of various physiological endpoints, such as cell membrane integrity and metabolic function. Several intracellular enzymes have been used as markers of physiological alterations in a cell. Lactate dehydrogenase (LDH) is commonly found in most cell types, and the LDH test is relatively easy to perform (27,28).

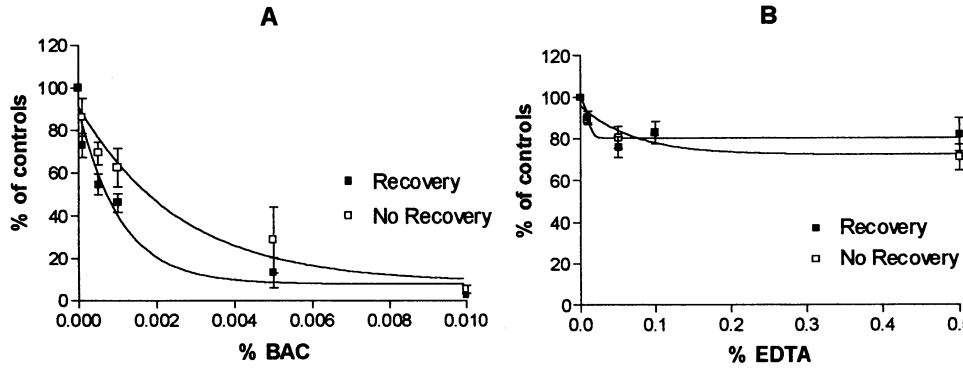

FIGURE 3. Reduction of WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) in Human Corneal Epithelial Cell Line Exposed for 5 min to Benzalkonium Chloride (BAC) (**A**) or to EDTA (**B**). Exposure without fetal bovine serum (FBS).

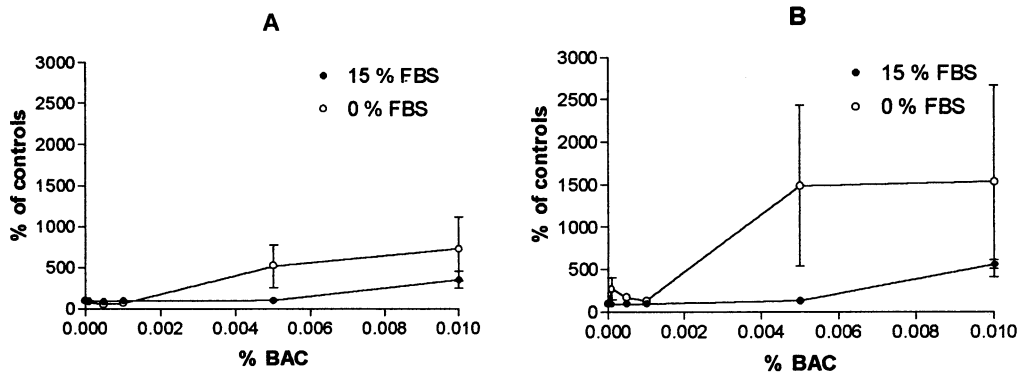

FIGURE 4. LDH (Lactate Dehydrogenase) Leakage in Human Corneal Epithelial Cell Line (**A**) and Rabbit Corneal Epithelial Primary Cells (**B**) Exposed to Benzalkonium Chloride (BAC) for 1 hr. FBS (fetal bovine serum).

The tetrazolium salt WST-1 test is based on the measurement of the function of mitochondrial succinate dehydrogenases (29). The cleavage of the tetrazolium salt WST-1 to formazan only occurs in the active mitochondria of living cells. Unlike MTT test, which is widely used to measure cell viability and proliferation, WST-1 test yields water-soluble cleavage products and, thus, does not require any additional solubilization steps before photometric measurements.

Although the LDH leakage test as an index of membrane integrity and the WST-1 test as an index of cell viability and metabolic function measure different physiological endpoints, they provide comparable results, especially in the case of EDTA-treated cells. In BAC-treated cells, however, the WST-1 test appeared to be an earlier indicator of cytotoxicity than the LDH leakage test. Table 2 presents *in vivo* and *in vitro* information of benzalkonium chloride found in the literature. Draize scores indicate that out of the possible 110 points, 1% BAC produces 56 points, 5% BAC produces 84 points and 10% BAC produces 108 points (30,31). The reported EC_{50} values evaluated by the MTT test for primary cells of rabbit corneal epithelium vary from 0.0005 to 0.0008% (w/v) (13,15,17). Our EC_{50} value for BAC-treated HCE cells (determined in serum-free conditions) in the WST-test was four times smaller than previous results in the MTT-test for the same cell line (32). In addition to the sensitivity of WST-test, the presence of serum in the medium may have caused these differences. After initial leakage of LDH (increase in LDH activity) in BAC-treated cells, there was a reduction of LDH

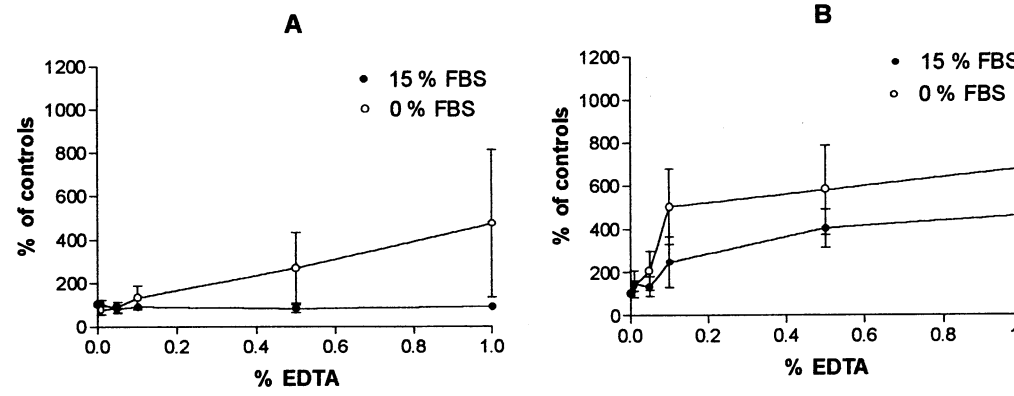

FIGURE 5. LDH (Lactate Dehydrogenase) Leakage in Human Corneal Epithelial Cell Line (**A**) and Rabbit Corneal Epithelial Primary Cells (**B**) Exposed to EDTA for 1 hr. FBS (fetal bovine serum).

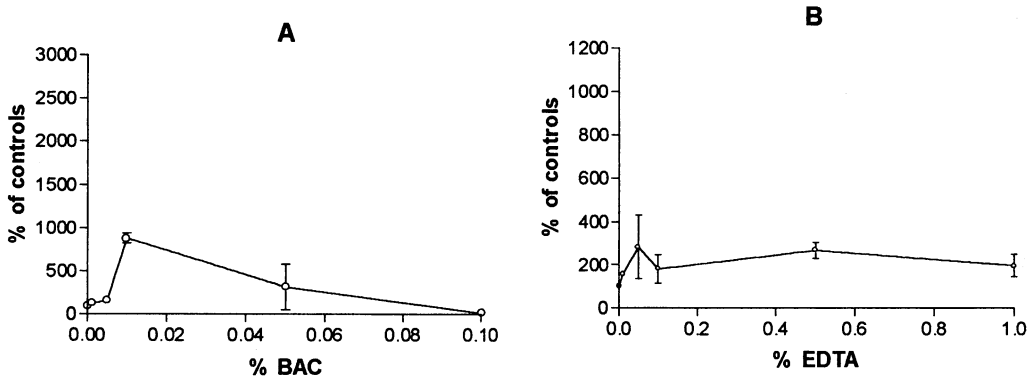

FIGURE 6. (LDH) Lactate Dehydrogenase Leakage in Human Corneal Epithelial Cell Line Exposed for 5 min to Benzalkonium Chloride (BAC) (**A**) or EDTA (**B**). Exposure without FBS (fetal bovine serum).

activity starting from 0.01% BAC concentration. This could be partly due to inactivation of LDH by BAC, because cationic surfactants can inactivate LDH isoenzymes (33). According to our experience, possible interactions of the test compounds with LDH and great variations in the LDH test reduce the reliability of the test in assessing the cytotoxicity of drugs.

The utilization of transformed or established cell lines has been criticized, because cell lines generally lack some of the specific biochemical and physiological functions characteristic of the target cell (34). Moreover, the use of primary cells as toxicity-screening systems has been considered more reliable in predicting the cytotoxicity *in vivo*, since primary cultures are believed to be more sensitive to a given concentration of test agents than the established or transformed cell lines (34). We did not find any statistically significant differences between RCE cell culture and HCE cell line in their responses to BAC and EDTA. In the comparison of the cytotoxicity in primary corneal cultures and in non-corneal cell lines, Borenfreund and Borreo (34) found that although early-passage rabbit corneal epithelial cells were more sensitive to a given concentration of test agents than the more established cell lines, the general ranking of test agents was similar for all cell types. The widely used rabbit

TABLE 2.
In Vivo and *in Vitro* Information of Benzalkonium Chloride (BAC)

In vivo* test** **Draize test** **MMAS	***In vitro* test** **EC_{50} (MTT test)** **(MEAN±SEM (%))**		**References**
	RCE	**HCE**	
1 % BAC 34.3/56.3**			30,31
5 % BAC 84			
10 % BAC 108			
	0.00070± 0.00001		13
	0.00080		15
	0.00051± 0.00004		17
		0.00468 ± 0.00051	32

*Modified Maximum Average Score, ** Two Different Results in the Same Laboratory. According to the Draize test, chemicals can be categorized as nonirritating (0.0), minimally irritating (0.1-15), mildly irritating (15.1-25), moderately irritating (25.1-50), severely irritating (50.1-80), or extremely irritating (80.1-110). RCE (Rabbit Corneal Epithelial Cells), HCE (Human Corneal Epithelial Cells).

corneal cell line SIRC has also been used to assess the cytotoxicity of various surfactants (35–37). However, SIRC has been reported to possess features that are more prominent to a fibroblastic cell type than an epithelial cell type (38). In the characterization study of HCE cell line used in the present study, it was shown to express epithelial cell characteristics (our unpublished data).

While most of the drugs are lost from ocular tissues and fluids in a few hours, anionic and cationic surfactants show retention (39). The surfactants are retained in the corneal epithelium, which may act as a reservoir for further penetration. This also affected our results. When the HCE cells were exposed to the cationic surfactant BAC for five minutes, washed once and then subjected to the WST-1 test immediately or after one-hour recovery time, less cytotoxic effects appeared in the cells that were not allowed to recover after the BAC treatment, which suggests a permanent binding of BAC on the cells. The binding of substances to biological membranes due to their physicochemical properties may affect the *in vitro* determinations (40). In our work, the 1 hr incubation in the medium after the removal of BAC seemed not to work for the cells as a time to recover, but as an extended exposure time. This phenomenon should be taken into consideration when the toxicity of BAC and other related surfactants is evaluated.

EDTA is a known calcium chelator. It is added to ophthalmic preparations for stability purposes. Traditionally, EDTA has been used for removal of the superficial calcific opacities and as an aid in the decontamination of the eye after splash of calcium hydroxide or lime (23). EDTA increases the permeability of the cornea (41). At low concentrations, EDTA has little effect other than what can be attributed to loss of calcium, as has been shown by studies on excised corneas, permeability, cytotoxicity, and ion flux (23). EDTA (0.1%) has been shown to be non-irritating *in vivo* with the rabbit blinking count test (42). Our *in vitro* studies support the *in vivo* findings that EDTA is less oculotoxic than BAC. This was also seen in the five min exposure, which corresponds better to the clinical treatments.

The protective nature of serum in the medium was also evaluated. It is well known that serum promotes the growth of all cell types. The disadvantages of serum are that it contains a mixture of unknown growth factors and its content varies batch by batch, which brings along additional variations in terms of cell growth and response to toxic chemicals (43). Furthermore, chemicals may bind to serum proteins and give an underestimation of cytotoxic effects. As expected, our results also show that toxic effects appear at lower test agent concentrations in serum-free medium than in serum-containing medium. We suggest that serum should be eliminated from a culture medium in cytotoxicity testing to avoid underestimation of the toxic effects of the test compounds.

In conclusion, our cytotoxicity results were similar in human corneal epithelial cell line and in rabbit primary corneal epithelial cell culture. As repeated establishment of fresh primary corneal epithelial cells is time-consuming and effortful, and leads to great heterogeneity, we conclude that the human corneal epithelial cell line is a better model for studies of the corneal toxicity of drugs. Even though *in vitro* cytotoxicity tests cannot be considered as a complete alternative to *in vivo* ocular irritation testing, these tests could be used in preliminary screening and in finding toxic ranges.

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